REMARKS

In response to the Office Action mailed June 3, 2003, Applicants have amended the claims, which when considered with the following remarks, is deemed to place the present application in condition for allowance. Favorable consideration of all pending claims is respectfully requested.

In the first instance, Applicants through the undersigned, thank Examiners Collins and Nelson for their time and consideration in granting a personal interview with the undersigned and representatives from CropDesign, NV on Tuesday, October 14, 2003. Applicants further thank Examiner Collins for the helpful suggestions and guidance in amending the claims. The Examiner indicated that the claims as amended herein would be favorably considered.

As discussed during the personal interview, the specification and claims have been amended to reflect the presently accepted nomenclature for Cyclin Dependent Kinases (CDKs). At the time the present application was filed, the A-type CDK in *Arabidopsis thaliana* was known as cdc2a. Since then, the nomenclature for CDKs has changed. Submitted herewith as Exhibit D is a review article by Joubes, J., et al., (2000) "CDK-related protein kinases in plants" *Plant Molecular Biology 43*:607-620. In this article, a new naming system for CDKs is proposed. In the case of A-type CDKs, members are identified by Arabic numerals allocated sequentially. Joubes, J., et al. (2000), page 68, second column. Table 1 of Joubes et al. indicates that the old name for the A-type CDK in *Arabidopsis thaliana* was Atcdc2a, as indicated in the present specification. Table 1 also shows that the name presently accepted by those of skill in the art for Atcdc2a is CDKA;1.

Thus, the specification has been amended to disclose at page 1, between lines 14-16: "[i]n this application, the Cyclin Dependent Kinase (CDK) nomenclature announced in Joubes, J. et al., (2000) *Plant Molecular Biology 43*:607-620 is also adopted. Thus,

CDC2aAt or CDC2a may also be referred to as its new designation CDKA;1, especially in the claims."

It is respectfully submitted that the amendment of the claims to recite CDKA;1 instead of cdc2a does not constitute the addition of new matter. *Arabidopsis thaliana* only has one A-type CDK, namely CDKA;1 (formerly known as CDC2a). Further, Applicants submit that there is precedent for amending a patent application to allow for changes in nomenclature. The Federal Circuit recently reviewed an application where the written description accompanying new claim language specified that the amendment was merely renaming the invention, in accordance with accepted scientific norms. Specifically, "IFN-alpha" was substituted for "leukocyte interferon." The court ruled that there was no new matter violation as the amendment merely substituted terminology. *See Schering Corp. and Biogen, Inc. v. Amgen, Inc.*, 22 F.3d 1347,1353-1354, 55 USPQ2d 1650, 1654 (Fed. Cir. 2000).

The Examiner has made final the previously-issued restriction requirement. Thus, claims 13-16 have been withdrawn from consideration and claims 1-12 and 17-31 are presently under examination.

Applicants thank the Examiner for indicating that certified copies of both PCT/EP99/02696 and EP 98201279.1 have not yet been received in the present application. Applicants are currently obtaining certified copies of these documents and will submit them forthwith.

The specification has been objected to because it does not contain an abstract of the disclosure. Submitted herewith is an abstract on a separate sheet as required under 37 C.F.R. §1.72(b).

Claim 24 has been objected to due to reciting both "grape" and "grapes". As presently amended, claim 24 recites "grape" and not both "grape" and "grapes." Withdrawal of the

objection to claim 24 is therefore warranted. Claim 24 has also been amended to delete recitation of chicory, cassava, asparagus, carrot, celery, lentils, broccoli, cauliflower, brussel sprout, artichoke, okra, squash, kale, and collard greens.

Claims 1-12 and 17-31 have been rejected under 35 U.S.C.§ 112, first paragraph, as allegedly violative of the written description requirement. The Examiner's position is that the claims cover a method for obtaining plants tolerant to abiotic stress by introduction of any nucleic acid molecule or regulatory sequence that results in the presence of a CDK protein that is not susceptible to inhibitory phosphorylation under abiotic stress conditions including sequences that encode or affect a variety of CDK proteins. According to the Examiner, the specification "describes and characterizes only one nucleic acid molecule that confers abiotic stress resistance upon introduction into a plant, i.e., a nucleic acid molecule encoding an *Arabidopsis* CDC2a protein wherein the tyrosine at position 15 is substituted to phenylalanine and the threonine at position 14 is substituted to alanine." June 3, 2002 Office Action, Page 4.

In response to the rejection of claims 1-12 and 17-31 under the written description requirement of 35 U.S.C. §112, first paragraph, and in order to advance prosecution of this application, claim 1 has been amended to recite: "[a] method for obtaining a plant with increased tolerance to drought or salt stress conditions, said method comprising: (a) introducing into one or more cells, tissue or organs of a plant, a nucleic acid molecule encoding a Cyclin Dependent Kinase (CDK) mutein operably linked to a promoter which functions in a plant cell, wherein the CDK mutein has a non-phosphorylatable amino acid residue in a position that corresponds to the tyrosine located at position 15 in the amino acid sequence of *Arabidopsis thaliana* CDKA;1, and wherein the CDK mutein comprises a PSTAIRE cyclin binding motif; (b) regenerating plants from the one or more cells, tissue or organs of (a); (c) exposing the regenerated plants of (b) to drought or salt stress conditions;

and (d) selecting a plant with increased tolerance to drought or salt stress conditions compared to a corresponding wild type plant." Support for the amendment to claim 1 may be found throughout the specification, e.g., page 4, lines 4-6; page 5, line 10, to page 7, line 23; page 9, lines 8-18; page 14, line 11 to page 16, line 4; page 19, line 22 to page 21, line 29; page 37, line 5 to page 38, line 7; page 28, line 31 to page 29, line 2; page 41, line 14, to page 42, line 14.

Claims 2, 3, and 4 have been canceled without prejudice. Claim 5 has been amended to recite: "[t]he method of claim 1 wherein the CDK mutein further comprises a non-phosphorylatable amino acid residue in a position that corresponds to the threonine located at position 14 in the amino acid sequence of CDKA;1." Support for the amendments to claim 5 may be found throughout the specification, e.g., page 9, lines 19-22. Claim 6 has been amended to recite: "[t]he method of claim 5, wherein the CDK mutein is free of phosphate groups at amino acid residues corresponding to the tyrosine at position 15 and the threonine at position 14, in the amino acid sequence of CDKA;1 of *Arabidopsis thaliana*. Support for the amendments to claim 6 may be found throughout the specification, e.g., page 9, lines 16-22. Claims 7-9 are cancelled by this amendment.

Claim 10 has been amended to recite: "[t]he method of claim 1 or 5, wherein the non-phosphorylatable amino acid residue is phenylalanine." Support for the amendments to claim 10 may be found throughout the specification, e.g., page 10, lines 15-18. Claim 11 has been amended to recite: "[t]he method of claim 5, wherein the non-phosphorylatable amino acid residue is alanine." Support for the amendments to claim 11 may be found throughout the specification, e.g., page 10, lines 18-23. Claim 12 has been canceled without prejudice by this amendment.

Claim 17 has been canceled without prejudice by this amendment. Claim 25 has been amended to recite:"[a] vector comprising a nucleic acid molecule encoding a Cyclin

Dependent Kinase (CDK) mutein wherein the CDK mutein comprises a phenylalanine at a position corresponding to residue 15 in *Arabidopsis thaliana* CDKA;1, or wherein the CKI mutein comprises an alanine and phenylalanine at positions corresponding to residues 14 and 15 respectively, in *Arabidopsis thaliana* CDKA;1, wherein said nucleic acid molecule is operably linked to a chimeric, tissue-specific, or abiotic stress-inducible promoter." Support for the amendment may be found throughout the specification, e.g., page 17, last line to page 18, line 23.

In view of the amendments to the claims as outlined above, it is respectfully submitted that the present invention is fully supported by the written description in the specification. The claims as presently amended recite a CDK mutein comprising specific amino acid substitutions which substituted amino acid residues are non-phosphorylatable. Further, the claims recite a CDK mutein having a conserved PSTAIRE cyclin-binding motif. As described in the specification on page 6, lines 1-17:

> While the findings described above have been obtained with the CDK protein CDC2a of *Arabidopsis thaliana*, the present invention can be performed with any CDK protein that is functional in plants, i.e., plant CDK proteins, functionally equivalent to the known CDC2a of Arabidopsis thaliana. With "plant CDK protein, functionally equivalent to the known CDC2a of *Arabidopsis thaliana*" is meant each CDK protein having a similar regulatory function as CDC2a of *Arabidopsis thaliana* in plants or plant cells respectively, e.g., having the PSTAIRE conserved cyclin binding motif, and the above-mentioned phosphorylatable tyrosine and threonine residues. Intensive cloning efforts have identified a large number of CDK proteins in diverse plant species, among which at least five types can be distinguished on the basis of their sequences (for a compilation see Seger, In: Plant cell proliferation and its regulation in growth and development. Bryant JA, Chiatante E, editors. Chichester: John Wiley & Sons (1997), 1-19). In the model plant, *Arabidopsis thaliana*, two CDKs, each belonging to a different family, have been characterized. One such example is the CDC2At gene, which contains the conserved PSTAIRE amino acid motif, and is constitutively expressed during the cell cycle at transcriptional and protein level.

Further, in a review by Mironov *et al.* (April 1999) *The Plant Cell, 11*:509-521 (a copy provided herewith as Exhibit A), the authors state in relation to CDKs that: "[t]he degree of evolutionary conservation is especially striking among eukaryotes, where progression through the successive phases of the cell cycle (S, G2, M and G1) in species as diverse as yeast and humans is driven by a common class of heterodimeric serine/threonine protein kinases." A similar conclusion is reached by Stals, H., and Inze, D., (August 2001) *TRENDS in Plant Science, 6* (8): 359-364, submitted herewith as Exhibit B, where it is disclosed: "[t]he main drivers of the cell cycle in yeast, mammals and plants are a class of highly conserved serine/threonine kinases known as cyclin-dependent kinases (CDKs)".

Thus, it is respectfully submitted, that nucleic acid molecules encoding CDK muteins and methods of using same to produce stress resistant plants, as presently recited in the claims, are sufficiently described in the specification in a way to reasonably convey to those skilled in the art at the time the application was filed, that Applicants were in possession of the invention. Withdrawal of the rejection of claims 1-12 and 17-31 under the written description requirement of 35 U.S.C.§ 112, first paragraph, is therefore respectfully requested by Applicants.

Claims 1-12 and 17 -31 have been rejected under 35 U.S.C.§112, first paragraph, as allegedly directed to non-enabled subject matter. According to the Examiner, the specification allegedly discloses only one method for obtaining plants tolerant to only one abiotic stress condition (salt). This method comprises introducing into *Arabidopsis* plants a nucleic acid sequence encoding an *Arabidopsis* CDC2a (CDKA;1) protein wherein the tyrosine at position 15 is substituted to phenylalanine and the threonine at position 14 is substituted to alanine. Further according to the Examiner, the nucleic acid sequence is operably linked to a CaMV 35 S promoter.

Applicants respectfully submit that the specification fully enables one skilled in the art to practice the invention as presently claimed in this amendment. While the specification *exemplifies* a particular method using a particular CDK mutein construct, the specification of the present application describes many different methods and compositions useful for practicing the present invention. As discussed hereinabove, by this amendment, claim 1 is presently limited to introducing into one or more cells, tissue or organs of a plant, a nucleic acid molecule encoding a Cyclin Dependent Kinase (CDK) mutein operably linked to a promoter which functions in a plant cell, wherein the CDK mutein has a non-phosphorylatable amino acid residue in a position that corresponds to the tyrosine located at position 15 in the amino acid sequence of *Arabidopsis thaliana* CDKA;1, and wherein the CDK mutein comprises a PSTAIRE cyclin binding motif. The specification clearly teaches that the amino acid residue correlating to the tyrosine located at position 15 in the amino acid sequence of *Arabidopsis thaliana* CDKA;1, is substituted with a non-phosphorylatable amino acid residue. Likewise, the specification clearly teaches that in a more preferred embodiment, both the amino acid residues correlating to the tyrosine located at position 15 and the threonine located at position 14 in the amino acid sequence of *Arabidopsis thaliana* CDKA;1, are substituted with non-phosphorylatable amino acid residues. Preferred embodiments include phenylalanine (claim 10) and alanine (claim 11) as the non-phosphorylatable amino acids.

Applicants also respectfully submit that the specification teaches plants having increased tolerance to many different abiotic stress conditions such as, e.g., water stress, anaerobic stress, osmotic or salt stress, temperature stress, stress caused by nutrients and/or pollutants. In response to the rejection, and in order to advance prosecution of this application, Applicants have amended the claims to recite methods for obtaining plants with increased tolerance to water or salt stress conditions. Example 2 of the specification

provides results of salt-tolerant plants comprising the compositions of, and produced by, the presently claimed invention. In addition, Applicants provide herewith at Exhibit C, a copy of Schuppler, U., et al., (June, 1998) "Effects of Water Stress on Cell Division and Cell-Division-Cycle 2-Like Cell-Cycle Kinase Activity in Wheat Leaves" *Plant Physiol. 117*:667-678. At page 668, first column, final sentence, the authors report: "[w]e report evidence that water stress has rapid effects on Tyr phosphorylation and activity of a Cdc2-like enzyme and propose that these contribute to reduced-cell division activity." More specifically, the paper teaches that inactivation of CDKA;1 by water stress in plants was associated with an increase in the proportion of Tyr-phosphorylated protein, whereas the amount of protein remained constant. *See* page 675, column 2, final paragraph. Further, at page 676, column 1, first full paragraph, the authors report that in the early response to water stress, there is an increase in Cdc2-phospo-Tyr and a decrease in Cdc2 activity which indicate that the balance of kinase and phosphatase activities that control Cdc2 Tyr phosphorylation is responsive to stress in the plant. Finally, at page 677, first full paragraph, the authors report: "[w]e propose that water stress induces a signal that increases the phosphorylation of Tyr at the active site of Cdc2 kinase and that this results in a predominance of the inactive form of the enzyme, with consequent inhibition of progression into mitosis, which is dependent on high activity of the enzyme." Applicants respectfully submit therefore, that the paper by Schuppler U., et al., submitted herewith as Exhibit C, evidences that those skilled in the art following the teaching provided in the present application, have also indicated that introduction of a nucleic acid molecule encoding a non-phosphorylatable CDK mutein into a plant will result in a plant tolerant to drought stress. Applicants respectfully submit therefore, that the presently claimed invention is fully enabled by the specification. In view of the amendments to the claims and the remarks hereinabove, withdrawal of the rejection of claims 1-12 and 17-31 under the enablement provision of 35 U.S.C. §112, first paragraph, is warranted.

Claims 1, 2, 3, 5, 6, 10, 11, 18, 25, 26, 27, 29 and 30 and claims dependent thereon have been rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, claims 1, 20, and 29 have been deemed indefinite in their recitation of "abiotic stress." The preamble to amended claim 1 recites: "a method for obtaining a plant with increased tolerance to drought or salt stress conditions." Claim 21 is presently canceled without prejudice. Claim 29 is presently amended to recite "the transgenic plant of claim 28 which displays increased tolerance to drought or salt stress, compared to the corresponding wild type plant."

The Examiner has deemed claim 1 indefinite due to recitation of "tolerant." In response to the rejection, claim 1 has been amended to recite in relevant part: "(d) selecting a plant with increased tolerance to drought or salt stress conditions compared to a corresponding wild type plant."

Claim 1 has also been deemed allegedly indefinite it reciting "a nucleic acid molecule or regulatory sequence" because it is unclear in what way a "regulatory sequence" would differ from "a nucleic acid molecule." As presently amended, claim 1 no longer recites "regulatory sequence."

The Examiner has found claim 2 allegedly indefinite it its recitation of "PSTAIR type." Claim 2 is canceled without prejudice by this amendment. Claim 1 has been amended to recite in relevant part: "wherein the CDK mutein comprises a PSTAIRE cyclin binding motif." Page 6, lines 6-7 of the specification, provide support for this amendment.

Claims 3, 5, and 6 are allegedly indefinite in the recitation of "CDC2a." Claim 3 is canceled without prejudice by this amendment. Claim 1 has been amended to recite in relevant part: "wherein the CDK mutein has a non-phosphorylatable amino acid residue in a position that corresponds to the tyrosine located at position 15 in the amino acid sequence of

Arabidopsis thaliana CDKA;1. Claim 5 has been amended to recite: "wherein the CDK mutein further comprises a non-phosphorylatable amino acid residue in a position that corresponds to the threonine located at position 14 in the amino acid sequence of *Arabidopsis thaliana* CDKA;1." Support for such amendments may be found throughout the specification, e.g., page 9, lines 8-14.

Claims 10 and 11 have been deemed allegedly indefinite in the recitation of "Y-15 →F15." Claim 11 has also been found allegedly indefinite in its recitation of "T-14 → A-14." As presently claim 10 no longer recites "Y-15 →F15." Claim 11 also no longer recites"T-14 → A-14."

Claim 18 is deemed allegedly indefinite due to the recitation of "UTR" which, according to the Examiner is an acronym whose meaning is unclear. Applicants respectfully direct the Examiner to page 8, lines 17 and 18 which disclose "3' UTR and/or 5' UTR coding regions." It is respectfully submitted that one skilled in the art at the time the application was first filed would have an understanding of the terms "3' UTR and/or 5' UTR coding regions" to mean "3' untranslated region and/or 5' untranslated coding regions."

Claim 25 is allegedly indefinite it reciting "the nucleic acid molecule of claim 20" because it lacks antecedent basis as claim 20 is directed to a method.. As presently amended, claim 25 recites: "[a] vector comprising a nucleic acid molecule encoding a Cyclin Dependent Kinase (CDK) mutein wherein the CDK mutein comprises a phenylalanine at a position corresponding to residue 15 in *Arabidopsis thaliana* CDKA;1, or wherein the CDK mutein comprises an alanine and phenylalanine at positions corresponding to residues 14 and 15 respectively, in *Arabidopsis thaliana* CDKA;1, wherein said nucleic acid molecule is operably linked to a chimeric, tissue-specific, or abiotic stress-inducible promoter."

Claim 26 is allegedly indefinite since it depends from a method claim. As presently amended, claim 26 recites: "a transgenic plant cell comprising the nucleic acid molecule of claim 25."

Claim 27 is allegedly indefinite in the recitation of "additional phenotypic characteristic." Claim 27 is canceled without prejudice by this amendment.

Claim 29 has been deemed indefinite in its recitation of "preferably." As presently amended, the term "preferably" is no longer recited in claim 29.

Claim 30 is allegedly indefinite in the recitation of "additional phenotypic characteristic." Claim 30 is canceled without prejudice by this amendment.

Claim 31 has been rejected under 35 U.S.C. § 101, as allegedly directed to non-statutory subject matter. As the Examiner has suggested, claim 31 is amended to indicate the hand of the inventor.

In view of the amendments to the claims as discussed above, withdrawal of the rejection of claims 1, 2, 3, 5, 6, 10, 11, 18, 25, 26, 27, 29 and 30 under 35 U.S.C. §112, second paragraph.

Claims 1-12, 17-19, 22-24 and 27 have been rejected under 35 U.S.C. §102(b) as allegedly anticipated by Hemerly et al. (1995) *EMBO J. 14*(16):3925-3936. Hemerly et al. has been cited for teaching a method comprising introducing into an *Arabidopsis* or tobacco plant cell a nucleic acid molecule that encodes a non-phosphorylatable *Arabidopsis* CDKA;1 mutein comprising a Y-15 $\rightarrow$ F-15 mutation and a T-14 $\rightarrow$ A-14 mutation operably linked to a constitutive CaMV 35 S promoter, and transgenic plants comprising the nucleic acid molecule.

The Examiner's position is that plants transformed with the same nucleic acid molecule as that claimed would have been inherently abiotic stress tolerant.

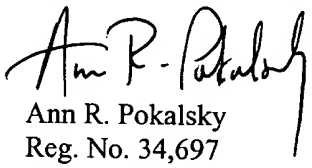
As presently amended, claim 1 recites the steps of regenerating plants from a cell(s), tissue or organs transformed with a nucleic acid molecule encoding the subject CDK mutein, exposing the regenerated plants to drought or salt stress conditions, and selecting a plant with increased tolerance to drought or salt stress conditions compared to a corresponding wild type plant. Support for the amendment of claim 1 to recite these steps may be found throughout the application, e.g., page 19, line 22 to page 21, line 29; page 37, line 5 to page 38, line 7; and page 28, line 31 to page 29, line 2. Applicants respectfully submit that Hemerly et al. do not teach or even suggest a method for obtaining a plant with increased tolerance to drought or salt stress conditions by introducing a nucleic acid molecule encoding the subject CDK mutein and which entails regenerating plants from a cell(s), tissue or organs transformed with a nucleic acid molecule encoding the subject CDK mutein, exposing the regenerated plants to water or salt stress conditions, and selecting a plant with increased tolerance to water or salt stress conditions compared to a corresponding wild type plant.

Anticipation under 35 U.S.C.§ 102 requires that the prior art reference disclose *every element* of the claims. *In re King,* 801 F.2d 1324, 1326, 231 USPQ 136, 138 (Fed. Cir. 1986). Thus, there must be no differences between the subject matter of the claims and the disclosure of the prior art reference. Stated otherwise, the reference must contain within its four corners adequate direction to practice the invention. The corollary of this rule is equally applicable. The absence from the reference of *any* claimed element negates anticipation. *Kolster Speedsteel AB v. Crucible Inc.*, 793 F.2d 1565, 1571, 230 USPQ 81, 84 (Fed. Cir. 1986).

The present invention differs from Hemerly et al. with respect to the novel features described above. As supported by the holding of *Kolster Speedsteel*, Hemerly et al. falls short of the statutory standard of 35 U.S.C. §102(b). Withdrawal of the rejection of claims 1-12, 17-19, 22-24 and 27 under 35 U.S.C. § 102(b) is therefore warranted.

In view of the forgoing amendments and remarks hereinabove, it is respectfully submitted that the present claims are in condition for allowance, which action is respectfully requested. The Examiner is invited to please telephone the undersigned if possible, in order to resolve any outstanding issues by e.g., an Examiner's amendment.

Respectfully submitted,



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Cyclin-Dependent Kinases and Cell Division in Plants—The Nexus

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INTRODUCTION

Cell division is one of the most conspicuous features of life, and thus several elements of the control of cell division are common to both prokaryotes and eukaryotes (Amon, 1998; Leatherwood, 1998). The degree of evolutionary conservation is especially striking among eukaryotes, where progression through the successive phases of the cell cycle (S, G2, M, and G1) in species as diverse as yeast and humans is driven by a common class of heterodimeric serine/threonine protein kinases. These kinases consist of a catalytic subunit, termed cyclin-dependent kinase (CDK), and an activating subunit, cyclin (reviewed in Nigg, 1995).

The first indication that this commonality might extend to the plant kingdom came with the identification of a plant protein immunologically related to the CDKs (John et al., 1989), a finding that was followed by the cloning of a cDNA fragment encoding a CDK-like protein from pea (Feiler and Jacobs, 1990). Subsequently, it became clear that several putative CDKs and cyclins are present in each plant species and that at least some of them are pertinent to our understanding of cell division control (Hemerly et al., 1995; Doerner et al., 1996).

Several questions nevertheless remain to be addressed. For example, which putative cyclins and CDKs are involved in cell division control in plants? What are their particular functions? How is their activity regulated? Here, we focus on molecular control of the cell cycle in higher plants and do not deal with the developmental and environmental control of cell division. For more information on these latter issues, the reader is referred elsewhere (Francis et al., 1998).

DIVERSITY OF CDKs AND CYCLINS IN PLANTS

Intensive cloning efforts over the past 7 years have identified a large number of CDK-like proteins (referred to as CDKs hereafter) in diverse plant species, among which at least five types can be distinguished on the basis of their sequences (see Segers et al., 1997; summarized in Table 1). The best-characterized plant CDKs belong to the A-type class. This class comprises kinases most closely related to the prototypical CDKs, yeast cdc2/CDC28 and animal CDK1 and CDK2, which share the conserved PSTAIRE motif in the cyclin binding domain. In addition to this large group of CDKs, several non-PSTAIRE CDKs have been described in plants. Some of them cannot be clearly affiliated with any other class of CDKs on the basis of sequence similarity. The situation may be even more complex in plant species that possess more than one representative of a given type of CDK, although it should be noted that not all plant species appear to possess all types of CDKs. For example, no homologs of the rice R2 kinase could be detected in Arabidopsis (Yamaguchi et al., 1998). Currently, only A and B classes of CDKs are well defined; other classes are represented only by one or two known members whose distribution in the plant kingdom remains unclear.

A similar situation exists with the cyclins. Numerous cDNAs encoding putative cyclins (referred to as cyclins hereafter) have been identified in a diverse range of plant species (for a compilation, see Renaudin et al., 1996). Arabidopsis alone possesses at least 15 cyclins. Analysis of the deduced peptide sequences in the conserved "cyclin box" has enabled the classification of these cyclins into nine groups: A1, A2, A3, B1, B2, D1, D2, D3, and D4, with the lettering scheme reflecting their similarities with the mammalian cyclins A, B, and D (Table 2; Renaudin et al., 1996; De Veylder et al., 1999). More recently, cyclins with similarity to mammalian cyclin C have been identified in rice and Arabidopsis, thus adding even greater complexity to efforts to classify plant cyclins.

The classification scheme described above, although helpful, does not necessarily reflect the functional properties of the cyclins. In particular, cyclins from groups A2, B1, D2, and D3 may comprise functionally distinct members, as judged by their subcellular localization and expression patterns (see below). In due course, the completion of the

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Table 1. Classification of CDKs in Plants

Class	Cyclin Binding Motif	Typical Phase Dependence[a]	Members Discussed	Closest Mammalian Homolog	Comments
A-type	PSTAIRE	Nonspecific	CDC2aAt CDC2aMs CDC2bMs CDC2aZm	CDK1, CDK2	Many complement yeast cdc2/CDC28 mutants; expressed in cycling cells and cells showing competence for division; high kinase activity in S, G2, and M
B-type	PPTALRE PPTTLRE	S/G2 G2/M	CDC2bAt CDC2fMs	Unknown Unknown	Do not complement yeast cdc2/CDC28 mutants; maximum kinase activity in M; expressed typically in dividing cells
Nonclassified CDKs	NFTALRE	G1/S?[b]	R2	CDK7 (CAK)	Phosphorylates CDKs and/or the large subunit of RNA polymerase II; complements *civ1/cak1* mutant in budding yeast
	PITAIRE SPTAIRE	Nonspecific Nonspecific	CDC2cMs CDC2eMs	CHED kinase? CDK8?	

[a] At the transcriptional level.
[b] Question marks denote uncertainty.

Arabidopsis genome sequencing project will provide definitive answers to questions regarding the profusion and diversity of plant CDKs and cyclins.

WHICH PLANT CDKs AND CYCLINS ARE AT WORK IN THE CELL CYCLE?

Lately, it has become increasingly clear that certain CDKs and cyclins in yeast and animals have nothing to do with cell division control. Thus, the time is ripe to ask the question, which of the many identified plant CDKs and cyclins are actually involved in regulating the cell cycle? There is now an extensive, albeit mainly circumstantial, body of evidence that at least some plant CDKs and their associated proteins function in cell cycle control. One of the strongest arguments is the ability of many of these proteins to substitute the functions of their yeast and animal homologs (Tables 1 and 2; Hata et al., 1991; Hemerly et al., 1992; Renaudin et al., 1994; Dahl et al., 1995; Meskiene et al., 1995; Setiady et al., 1995; Soni et al., 1995; Day et al., 1996; Ito et al., 1997; Sundaresan and Colasanti, 1998). Although these data might appear to be conclusive, it should be noted that animal B and C cyclins are known to complement G1 cyclin deficiency in yeast despite the fact that there is no indication that they play a role in G1 progression (in the case of cyclin B) or any aspect of cell division (cyclin C) in animals. The consistently observed correlation between cell division and the expression patterns of many plant cyclins and CDKs is, similarly, both supportive and circumstantial.

More compelling data—accelerated progression through mitosis, including a rapid disintegration of the preprophase band (PPB), nuclear envelope breakdown, and chromosome condensation—have been obtained upon injection of active CDK complexes from metaphase plant cells into *Tradescantia* stamen hair cells (Hush et al., 1996). The composition of the complexes used in these studies, however, is unknown.

Currently, experiments in plants support relevance for cell division control only for CDC2aAt, CDC2bAt, and CYCB1;1 and by extrapolation for their orthologs from other species. Hemerly et al. (1995) have demonstrated that downregulation of CDC2aAt activity in plants is sufficient to compromise the rate of cell proliferation and other aspects of cell division, such as the orientation of cell division planes and cell size control. Because such downregulation does not affect the relative duration of G1 and G2, CDC2aAt probably functions in both the G1-to-S and G2-to-M transitions. In a similar way, we have shown that downregulation of B-type CDKs in transgenic plants lengthens the relative duration of G2, thus implicating these kinases in the progression through G2 (V. Mironov, A. Porceddu, J.-P. Reichheld, and D. Inzé, unpublished results). On the other hand, Doerner et al. (1996) have shown that cell proliferation in Arabidopsis roots can be boosted by ectopic expression of the CYCB1;1 cyclin. This work demonstrates that CYCB1;1 might be a limiting factor for cell division in Arabidopsis, although the phase of the cell cycle at which it operates was not identified (Doerner et al., 1996).

There is no evidence for cell cycle functions of PITAIRE or SPTAIRE CDKs. Moreover, the expression pattern of an Arabidopsis PITAIRE kinase closely related to CDC2cMs argues

against the involvement of this group of CDKs in cell division control because no expression associated with actively dividing cells was detected by in situ hybridization (V. Mironov, R.M. de Pinho Barroco, and D. Inzé, unpublished results).

HOW DOES CDK ACTIVITY CHANGE THROUGH THE CELL CYCLE?

Routinely, CDK activity is assessed by histone H1 phosphorylation, and substantial biochemical evidence for the presence of CDK activity in diverse plant cells has been generated by using histone H1 as a substrate for CDK complexes purified by $p13^{suc1}$ and $p9^{CKS1Hs}$ affinity selection (Jacobs, 1995). However, these data are mainly inconclusive and difficult to interpret because the composition of the complexes is in the best case guesswork, given the ability of a number of distinct kinases, including A- and B-type CDKs, to bind $p13^{suc1}$ and $p9^{CKS1Hs}$. In only a few instances has CDK activity been traced down to a specific CDK or cyclin that has previously been identified by cloning. In particular, histone H1 kinase activity specifically associated with A-type CDKs has been analyzed in partially synchronized suspension cells of alfalfa (Bögre et al., 1997; Magyar et al., 1997), Arabidopsis, and tobacco (J.-P. Reichheld and D. Inzé, unpublished results). Most of these data consistently demonstrate high kinase activity in S, G2, and M phases, with a pronounced recession in G1.

By contrast, the activity of B-type kinases is prominently linked to mitosis. We have used specific antibodies against CDC2bAt to immunoprecipitate the histone H1 kinase activity associated with B-type CDKs in partially synchronized

Table 2. Classification of Cyclins in Plants

Class	Typical Phase Dependence[a]	Members Discussed[b]	Original Name	Comments
A1	S/G2/M	Zeama;*CYCA1;1* Nicta;*CYCA1;1*	*cycIIZm* *ntcyc25*	Zeama;*CYCA1;1* triggers frog oocyte maturation; Nicta;*CYCA1;1* rescues G1 cyclin deficiency in yeast
A2	S/G2/M	Nicta;*CYCA2;1* Medsa;*CYCA2;1*[c]	*ntcyc27* *cycMs3*	Medsa;*CYCA2;1* expression suppresses the α-pheromone–induced cell cycle arrest in yeast; Medsa;*CYCA2;1* and Nicta;*CYCA2;1* complement G1 cyclin deficiency in yeast
A3	S/early G2	Catro;*CYCA3;1*	*CYS*	Catro;*CYCA3;1* rescues G1 cyclin deficiency in yeast
B1	G2/M	Arath;*CYCB1;1* Arath;*CYCB1;2* Catro;*CYCB1;1* Nicta;*CYCB1;2* Zeama;*CYCB1;1* Zeama;*CYCB1;2* Glyma;*CYCB1;1*	*cyc1At* *cyc1bAt* *CYM* *ntcyc29* *cyclaZm* *cyclbZm* *S13-6*	Arath;*CYCB1;1*, Zeama;*CYCB1;1*, Zeama;*CYCB1;2*, and Glyma;*CYCB1;2* trigger frog oocyte maturation; Arath;*CYCB1;2*, Catro;*CYCB1;1*, and Nicta;*CYCB1;2* rescue G1 cyclin deficiency in yeast
B2	G2/M	Arath;*CYCB2;2* Zeama;*CYCB2;1* Medsa;*CYCB2;2*	*cyc2bAt* *cycIIIZm* *cycMs2*	Zeama;*CYCB2;1* triggers oocyte maturation; Medsa;*CYCB2;2*-immunoprecipitated kinase activity is maximal in G2
D1	Unknown	Arath;*CYCD1;1*	*cyclin δ1*	Rescues G1 deficiency in yeast; associates with CDC2aAt in the two-hybrid system
D2	Nonspecific	Arath;*CYCD2;1* Nicta;*CYCD2;1*	*cyclin δ2*	Arath;*CYCD2;1* rescues G1 deficiency in yeast; expression sucrose inducible; Nicta;*CYCD2;1* transcript peaks during M
D3	Nonspecific	Arath;*CYCD3;1* Medsa;*CYCD3;1* Nicta;*CYCD3;1* Nicta;*CYCD3;2*	*cyclin δ3* *cycMs4*	Arath;*CYCD3;1* and Medsa;*CYCD3;1* rescue G1 deficiency in yeast, expressed in only a subset of proliferating cells; Arath;*CYCD3;1* cytokinin inducible interacts with Rb and ICK1; Nicta;*CYCD3;1* transcript peaks during M
D4	Unknown	Arath;*CYCD4;1*		Expression sucrose inducible; expressed during lateral root primordia formation

[a] At the transcriptional level.
[b] Nomenclature according to Renaudin et al. (1996).
[c] Expressed in a nonspecific manner.

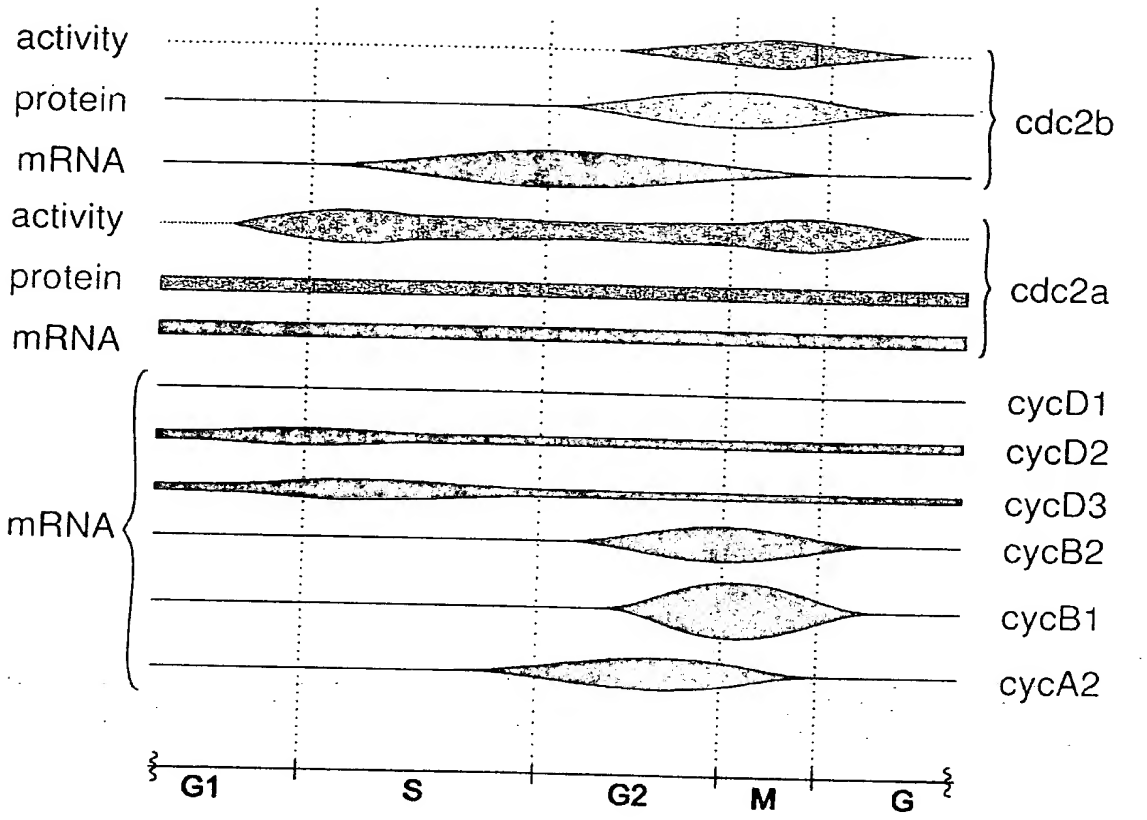

Figure 1. Control of Cell Cycle Genes in Arabidopsis.

Expression of Arabidopsis cyclins, CDKs, and CDK activities (using histone H1 as substrate) over the course of the cell cycle. The thickness of the filled areas qualitatively reflects the level of mRNA, protein, or activity, as indicated.

suspension cells and have found that the activity associated with CDC2bAt in Arabidopsis and the cognate protein in tobacco peak in the early M phase (J.-P. Reichheld and D. Inzé, unpublished results). Similarly, the activity of CDC2fMs sharply peaks in mitosis in partially synchronized alfalfa cells, albeit somewhat later (Magyar et al., 1997). The activity profiles of A- and B-type CDKs are illustrated in Figure 1.

It is still to be seen which cyclins contribute to all of these activities. Currently, the information is limited to the demonstration that histone H1 kinase activity peaks in G2 in complexes immunoprecipitated with antibodies against the alfalfa cyclin CYCB2;2 (Magyar et al., 1997).

There are two indications that biochemically distinct histone H1 kinases, characterized by their inability to bind to an affinity $p13^{suc1}$ matrix, may be activated during DNA replication in plant cells. Indeed, a histone H1 kinase isolated from endoreduplicating maize endosperm cells by virtue of its binding to the human E2F and adenovirus E1A proteins (Grafi and Larkins, 1995) proves to be almost absent from mitotically cycling endosperm cells. This kinase cross-reacts with an antibody against the A-type maize CDK CDC2aZm, but it does not bind $p13^{suc1}$. In addition, anti-human cyclin A antibodies precipitate a histone H1 kinase (of unknown identity) from alfalfa cells very early in S phase, a kinase that is also not recoverable by $p13^{suc1}$ affinity (Magyar et al., 1993).

No G1-specific CDK activities have been described in plants. In mammals, the G1 kinases consist of CDK4 or CDK6 associated with the D cyclins (Pines, 1996a). Biochemically, these CDKs differ considerably from the other CDKs in that they do not bind $p13^{suc1}$, and histone H1 is a very poor substrate. Instead, the retinoblastoma protein (pRB), a key regulator of the G1 transition, is the preferred substrate. The cloning of maize cDNAs coding for pRB-like

proteins (reviewed in Gutierrez, 1998) may provide the necessary tool for the detection of G1-specific CDK activities in plants.

WHAT DO WE KNOW ABOUT THE MOLECULAR MECHANISMS OF REGULATION?

In yeast and animals, CDK activity is regulated at several levels, including expression, differential subcellular localization, phosphorylation, proteolysis, and interaction with regulatory proteins. Below, we summarize our current knowledge of these events in plants.

Expression of CDKs and Cyclins

The expression of plant CDKs and cyclins has been studied rather extensively at the level of transcript accumulation. As a result, we know now that some plant CDKs cycle, as do many plant cyclins (Tables 1 and 2 and Figure 1). In particular, all the B-type kinases analyzed so far accumulate transcripts preferentially either in S and G2 or in G2 and M phases (Fobert et al., 1994, 1996; Segers et al., 1996; Magyar et al., 1997; Umeda et al., 1999). In contrast, cell cycle phase–independent expression is typical of the majority of the plant A-type CDKs (Martinez et al., 1992; Hemerly et al., 1993; Magyar et al., 1993, 1997; Fobert et al., 1996; Segers et al., 1996; Setiady et al., 1996; Sauter, 1997; Umeda et al., 1999).

The expression profiles of plant CDKs other than A or B type have drawn much less attention. The transcript of the rice CDK R2 is more abundant in G1 and S in partially synchronized rice suspension cells (Sauter, 1997) but is rather constant in rice root meristems (Umeda et al., 1999). The expression of CDC2cMs and CDC2eMs in partially synchronized alfalfa suspension cells remains constant throughout the cell cycle (Magyar et al., 1997).

As it is in animals, the phase-dependent expression of A- and B-type cyclins in plants is under transcriptional control. Moreover, there seems to be a fair degree of correlation between the temporal expression pattern and the cyclin class as defined by primary structure (Table 2; Fobert et al., 1994; Kouchi et al., 1995; Meskiene et al., 1995; Setiady et al., 1995; Reichheld et al., 1996; Segers et al., 1996; Shaul et al., 1996; Ito et al., 1997; Sauter, 1997; Lorbiecke and Sauter, 1999). Interestingly, the cyclin CYCA2;1 from alfalfa, related to the A2 group, is nevertheless expressed uniformly throughout the cell cycle and has consequently been proposed to play a role in G1 (Meskiene et al., 1995). In terms of the mechanisms of the G1 phase transition in plants, it is important to find out whether functional homologs of Medsa;CYCA2;1 are present in other species.

The majority of D-type cyclins in both plants and animals manifest fairly constant expression levels throughout the cell cycle (Dahl et al., 1995; Soni et al., 1995; Doonan, 1998). Plant D cyclins, by analogy with their animal homologs, have been proposed to control the G1 progression in response to growth factors and nutrients (Dahl et al., 1995; Soni et al., 1995). Unexpectedly, cyclins CYCD2;1 and CYCD3;1 from tobacco are found to be expressed predominantly during the G-to-M transition (Sorrell et al., 1999), suggesting that D-type cyclins in plants may also be involved in mitotic events.

Relatively little is known regarding the degree to which the protein levels of plant cell cycle genes follow the transcriptional patterns described above. The protein levels of A-type CDKs are rather stable throughout the cell cycle (Bögre et al., 1997; Magyar et al., 1997; Mews et al., 1997; J.-P. Reichheld and D. Inzé, unpublished results). The protein levels of B-type CDKs clearly peak in M phase (Magyar et al., 1997; Umeda et al., 1999; J.-P. Reichheld and D. Inzé, unpublished results).

The only relevant information regarding expression of

Table 3. Intracellular Location of Plant CDKs and Cyclins[a]

Location	A-Type CDKs	CYCA1;1	CYCB1;1	CYCB1;2	CYCB2;1
Interphase cytoplasm	±	+	±	+	−
Interphase nuclei	+	±	+	±	+
Prophase nuclei	+	+	+	+	+
Preprophase band	+	+	−	+	−
Mitotic spindle	+	+	−	+	−
Condensing chromosomes	−	−	−	+	−
Nuclear envelope	−	−	−	+	−
Phragmoplast	+	+	−	+	+
Interphase cortical microtubules	−	+	−	−	−

[a](+), strong labeling; (±), weak labeling; (−), undetectable labeling.

plant cyclins is provided by Mews et al. (1997), who used indirect immunofluorescence to localize four mitotic cyclins in the A1, B1, and B2 groups in maize root tip cells (Table 3). Because the signals obtained through immunolocalization may reflect epitope accessibility rather than actual protein levels, data of this kind should be interpreted with caution. Nevertheless, the results seem to confirm the prevalence of the cyclins in G2 and M and further suggest their persistence (with the exception of CYCB1;2) well into telophase. In this regard, it is worth noting that cyclins with specific functions in the completion of mitosis have recently been identified in yeast (Aerne et al., 1998).

Subcellular Localization

A steadily accumulating body of evidence points to the control of subcellular localization of a number of essential proteins, particularly CDC2, cyclin B, cyclin D, CDC25, and CDC6, as an important mechanism of cell cycle control in eukaryotes (Pines, 1999). In plants, this aspect of regulation has been addressed only for A-type CDKs and four mitotic cyclins (Table 3). A-type CDKs, when assayed by indirect immunofluorescence, are predominantly found in the interphase and early prophase nucleus in maize, alfalfa, and Arabidopsis, and to a lesser extent in the cytoplasm (Colasanti et al., 1993; Mews et al., 1996, 1997; Bögre et al., 1997; Stals et al., 1997).

During mitosis, A-type CDKs have been found in association with a number of cytoskeletal structures, such as the PPB, spindle, and phragmoplast. They also transiently interact with the chromosomes at the metaphase–anaphase transition in alfalfa (Stals et al., 1997) but apparently not in maize (Mews et al., 1997). The cytoplasmic labeling progressively declines as the cells of maize root tips exit the mitotic cycle and differentiate. However, the cognate proteins persist in the nuclei through all the developmental zones, including in differentiated cells (Mews et al., 1996). This observation may indicate that nuclear localization renders A-type plant CDKs less susceptible to proteolysis. However, Bögre et al. (1997) have observed that comparable amounts of A-type CDKs are present in the cytoplasmic and nuclear fractions of alfalfa cells in S phase, whereas the proteins are detectable by immunofluorescence only in the nucleus of the same cells. This observation suggests that epitope accessibility of plant CDKs may be influenced by subcellular localization.

The pioneering work of Mews et al. (1997) provides the first piece of data on the subcellular localization of plant cyclins (Table 3). All four cyclins display unique and dynamic patterns of localization, demonstrating that the functions of the numerous plant cyclins are not redundant. Particularly striking is the difference between the two B1 cyclins: whereas CYCB1;1, like CDC2Zm, is predominantly nuclear, CYCB1;2 localization closely resembles that of human cyclin B1 in that this cyclin is relocated to the nucleus in prophase and degraded in anaphase. Nuclear relocation in prophase has also been observed for CYCA1;1, which is at odds with the nuclear localization of animal cyclin A. These results clearly show that the functions of plant cyclins cannot be deduced from sequence similarity with their animal counterparts.

Formation of CDK/Cyclin Complexes

Whereas our knowledge of the expression of CDKs and cyclins in plants is already quite substantial, disappointingly little functional data exist regarding the CDK/cyclin complexes. First of all, we do not know whether plant CDKs are dependent on cyclins. Computer-assisted modeling of the three-dimensional structure of CDC2aAt (R. Abagyan, unpublished data) based on coordinates of the human CDK2 model (De Bondt et al., 1993) suggests that the plant kinase should be as cyclin dependent as the human enzyme. The only supporting experimental evidence, however, is circumstantial: on the one hand, Bögre et al. (1997) found that protein fractions from alfalfa extracts corresponding to monomeric CDKs are essentially devoid of kinase activity, as measured by histone H1 phosphorylation; on the other hand, alfalfa protein complexes immunoprecipitated with antibodies against the human cyclin A or alfalfa cyclin CYCB2;2 exhibit appropriate histone H1 kinase activity (Magyar et al., 1993, 1997).

Not a single active CDK/cyclin complex has been reliably identified in plants. The results of immunolocalization of CDC2Zm and mitotic cyclins in maize suggest several possible combinations (see Table 3), but these data fall short of proof. Two approaches pursued recently in our laboratory are beginning to shed light on the CDK/cyclin complexes of Arabidopsis. First, we have identified a number of proteins capable of interacting with CDC2aAt by using the two-hybrid system, including CYCD1;1 (De Veylder et al., 1997a) and CYCD4;1 (De Veylder et al., 1999). Nevertheless, it still has to be proven that such CDC2aAt/CYCD complexes are actually formed and active in plant cells. Indeed, some of the complexes formed by animal CDKs and cyclins, in particular complexes of cyclin D with CDK2 and CDK5, are known to be inactive (Ewen et al., 1993; Xiong et al., 1997).

Second, a procedure has been developed in our laboratory to purify active kinase complexes from Arabidopsis cells that contain selectively either CDC2aAt or CDC2bAt, whereby CYCB1;1 and CYCB2;2 were found to copurify preferentially with CDC2bAt and CDC2aAt, respectively (H. Stals and P. Casteels, unpublished data). Many more complexes will soon be characterized, but given the plethora of cyclins in plants, it may take some time to achieve a comprehensive overview of the system. The persistence of "orphan" cyclins in the more thoroughly characterized mammalian systems would seem to substantiate this caveat (Pines, 1996a).

Interacti n with Other Cell Cycl Regulators

Several noncyclin proteins have been found in complexes with CDKs. In particular, the family of evolutionarily conserved CKS proteins (for cyclin-dependent kinase subunit) is required for progression through the cell cycle in yeast and vertebrates, although the molecular mechanisms by which these proteins act remain elusive. Crystallographic and biochemical analyses suggest that CKS proteins act as docking factors for positive and negative regulators of CDKs (Pines, 1996b).

A plant CKS homolog, CKS1At, has been isolated through the use of a two-hybrid system using CDC2aAt as bait (De Veylder et al., 1997b). The *CKS1At* gene is functional in yeast, and its gene product associates with both A- and B-type Arabidopsis CDKs in vivo (in yeast) and in vitro. In situ hybridization analysis (Jacqmard et al., 1999) further reveals that *CKS1At*, together with *CDC2aAt* and *CDC2bAt*, is strongly transcribed in actively dividing tissues, suggesting that these proteins may also interact in plants. The presence of *CKS1At* expression in a number of polyploid tissues where *CDC2aAt* and *CDC2bAt* transcripts are present at very low levels or are absent (Jacqmard et al., 1999) indicates that CKS1At may play a role in the endocycle. It is conceivable that CKS1At is required for the functioning of a yet to be identified CDK of Arabidopsis, presumably one that is involved in the process of endoreduplication.

Much attention has been focused of late on a group of proteins in yeast and animals known as CDK inhibitors (CKIs). These proteins inhibit cell cycle progression through their association with CDK complexes (Nakayama and Nakayama, 1998). A first plant gene (*ICK1*) with limited sequence similarity to mammalian CKIs was isolated by using a two-hybrid system with the CDC2aAt protein as bait (Wang et al., 1997). Two additional putative plant CKIs have since been isolated in a similar way in our laboratory. Remarkably, all these proteins show only modest sequence similarity to the human $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ inhibitors. This similarity is restricted to a stretch of 30 amino acids located at the C terminus, which has been found crucial for the interaction of ICK1 with CDC2aAt and also CYCD3;1 (Wang et al., 1998). The remainder of the plant protein sequences has no similarity to any other protein in the public databases. Despite that, the results of Wang et al. (1998) show that the region adjacent to the conserved C terminus is, as in the animal counterparts, involved in the interaction with the cyclin (CYCD3;1 in this case). Recombinant ICK1 at nanomolar concentrations inhibits 80% of the total CDK activity (measured with the histone H1 kinase assay) recovered from Arabidopsis extracts by using $p13^{suc1}$ affinity selection (Wang et al., 1997). The failure to inhibit CDK activity completely is most probably due to the specificity of ICK1 for A-type CDKs (L. De Veylder and D. Inzé, unpublished results). Transcriptional induction of *ICK1* by abscisic acid suggests that ICK1 may mediate the cytostatic effect of abscisic acid in plants (Wang et al., 1998).

The in vivo function of the plant CKI-like proteins has still to be determined. The current experimental evidence indicating that CKIs may be deployed in plant cell cycle control is limited to two circumstantial observations. Grafi and Larkins (1995) have shown that cells of maize endosperm undergoing endoreduplication contain an unidentified active inhibitor of the histone H1 kinase activity of mitotically dividing endosperm cells. Bögre et al. (1997) analyzed histone H1 kinase activity of nuclear and cytoplasmic CDKs purified from synchronized alfalfa cells either by immunoprecipitation (A-type CDKs) or by $p13^{suc1}$ binding. The observed differences in the activity profiles suggest the presence of a thermolabile inhibitor, predominantly cytoplasmic, with higher affinity for nuclear S phase CDKs. Although in the absence of some essential controls this interpretation cannot be regarded as definitive, the observed phenomenon provides a promising assay for the biochemical identification of the presumed inhibitor.

CDK Phosphorylation

Considerable progress has been achieved lately in the analysis of the post-translational regulation of CDK/cyclin complexes in plants. Zhang et al. (1996) presented the first direct evidence for the phosphorylation of CDKs as a control mechanism in plants. Tobacco pith parenchyma and *Nicotiana plumbaginifolia* suspension-cultured cells, arrested in G2 by the absence of cytokinin, contain CDK complexes with both reduced kinase activity and high phosphotyrosine content. Resumption of the cell cycle upon addition of cytokinin, however, results in tyrosine dephosphorylation and kinase reactivation. The in vitro treatment of the complexes from cytokinin-depleted cells with the yeast cdc25 phosphatase, highly specific for the Tyr15 of CDKs, similarly leads to their dephosphorylation and activation, implicating the Tyr15 residue as the most probable target of the inhibitory phosphorylation.

Given that Tyr15 is almost universally conserved in plant CDKs, this type of phosphorylation-dependent regulation might well prove to be common in plants. Moreover, the requirement for cytokinin in *N. plumbaginifolia* cells can be completely alleviated by expression of the *cdc25* gene from fission yeast (John, 1998), thus suggesting the triggering of Tyr15 dephosphorylation as the only essential function of cytokinins in the plant cell cycle. The *cdc25* gene has also been expressed in transgenic tobacco plants and in cultured roots of tobacco, and in both cases the cells were found to divide at a reduced size (Bell et al., 1993; McKibbin et al., 1998). This observation further supports the importance of Tyr15 phosphorylation in the timing of mitosis in plants. Tyr15 phosphorylation also has been recently implicated in water stress responses in wheat (Schuppler et al., 1998). The identity of the CDK(s) subjected to inhibitory phosphorylation in all these cases, however, remains unknown.

The question of the function of Tyr15 phosphorylation has been approached from a different angle by Hemerly et al. (1995), who produced transgenic Arabidopsis and tobacco plants expressing the double T14A/Y15F mutant of CDC2aAt that is thought to be constitutively active. These plants, unlike those expressing *cdc25*, develop normally, except for some tendency toward a reduced apical dominance, but unfortunately they have not been analyzed cytologically. These results suggest either that CDC2aAt is not a substrate of the cytokinin-mediated control of Tyr phosphorylation or, more likely, that there are additional targets, presumably partially redundant with CDC2aAt. The much sought-after enzymes responsible for the phosphometabolism of T14/Y15 in plant CDKs are still awaiting discovery.

The majority of animal CDKs need to be phosphorylated by the so-called CDK-activating kinases (CAKs) for full activation (Harper and Elledge, 1998). Although it is not known whether this type of phosphorylation event is necessary to activate plant CDKs, the kinase CAK1At from Arabidopsis, which is only distantly related to the animal CAKs, has been demonstrated to possess CAK activity toward human CDK2/cyclinA complexes and to complement CAK-deficient mutants in both budding and fission yeast (Umeda et al. 1998). Similar to the budding yeast CAK1 kinase, CAK1At is not cyclin dependent.

There is an indication that the mechanisms of CDK activation may differ between monocotyledonous and dicotyledonous species. Yamaguchi et al. (1998) have found that the rice CDK R2, 50% identical to the animal CAK kinase CDK7, complements CAK deficiency in budding (but not fission) yeast and phosphorylates in vitro the rice CDC2Os1 and the human CDK2 with specificity identical to that of the human CAK. This observation is in conflict with a previous report that R2 has no CAK activity and instead phosphorylates efficiently the C-terminal domain of the large subunit of RNA polymerase II (Umeda et al., 1998).

Proteolytic Degradation

In the course of the past few years, controlled proteolysis has come into prominence as one of the most essential mechanisms underlying cell cycle transitions in eukaryotes (Peters, 1998). Very little is known about this aspect of cell cycle control in plants, primarily because the protein levels of plant cell cycle regulators have not been adequately characterized. From what is currently known, we can only surmise that certain B-type CDKs, for example, CDC2bAt, may be subject to proteolytic removal in the S and early G2 phases, given the considerable delay in the accumulation of the protein compared with the transcript (Figure 1).

Nevertheless, there is little doubt that this form of control exists in plants because (1) the ubiquitin-dependent proteolysis system is present in plant cells, and the expression of some of its elements has been linked to cell proliferation (Plesse et al., 1998); (2) plant A- and B-type cyclins feature the so-called destruction box, a hallmark of ubiquitin-mediated degradation, which has now been found sufficient for cell cycle–dependent protein instability in tobacco suspension–cultured cells (Genschik et al., 1998); and (3) PEST sequences, rich in proline (P), glutamate (E), serine (S), and threonine (T), are also portents of protein degradation and are found in both plant CDKs and cyclins.

In animals and yeast, two multisubunit E3 ubiquitin ligases, SCF and APC, have been found essential for the degradation of a number of cell cycle proteins, including cyclins and CKIs (Peters, 1998). Although homologs of a number of eukaryotic proteins that are related in function to SCF and APC have been described in plants (Leyser et al., 1993; Ingram et al., 1997; Luo et al., 1997; del Pozo et al., 1998; Porat et al., 1998; Ruegger et al., 1998; Xie et al., 1998), their destructive function and role in the cell cycle remain entirely speculative. Intriguingly, two of the plant homologs, AXR1 and TIR1, are implicated in auxin responses (Leyser et al., 1993; Ruegger et al., 1998). This observation raises the attractive possibility that auxin promotes cell division by triggering degradation of CKIs. However, a word of caution is necessary here because homologs of, for instance, TIR1 are involved in a diverse range of functions not necessarily related to the cell cycle.

Although the evidence for protein degradation as a universal mechanism in cell cycle control is accumulating, differences in the mechanisms of degradation of cell cycle proteins in plants compared with other eukaryotes are also anticipated. For example, some maize cyclins that bear a destruction box have been found to be resistant to proteolysis in anaphase (Mews et al., 1997). This finding implies the existence of an active mechanism selectively protecting plant cyclins against proteolysis in M phase.

FUNCTIONS?

The question mark in the title of this section is indeed necessary. Whereas a considerable amount of data implicates plant cyclins and CDKs in cell division control (as discussed above), the links between particular proteins and specific events during the cell cycle remain elusive. The subcellular localization of CDKs and cyclins provides some hints as to their potential functions (Table 3). Complexes of A-type CDKs and B1 cyclins of the CYCB1;2 subtype, for example, are very probably responsible for PPB disintegration, given that they both associate transiently with the PPB immediately beforehand (Hush et al., 1996). These same cyclins, but not the A-type CDKs, colocalize with the condensing chromosomes and the nuclear envelope before its breakdown and thus may be involved in the two processes.

By contrast, cyclin A1, in complexes with a succession of various CDK partners, may well control microtubule dynamics, as suggested by its association with all appropriate structures throughout the cell cycle. On the basis of their spatial (A-type) or temporal (B-type) expression patterns, neither A- nor B-type CDKs qualify as potential partners of cyclin A1 during the early interphase. This conclusion further invokes the presence of additional types of CDKs in the control of the plant cell cycle. Finally, A-type CDKs in complexes with cyclins B1 (CYCB1;1 subtype) and B2 are expected to phosphorylate nuclear proteins.

Growing evidence suggests that pRB-like proteins in plants might be among nuclear targets of plant CDKs. The pRB is central to the regulation of the G1-to-S transition in mammals. Phosphorylation of pRB by cyclin D- and cyclin E-dependent kinases renders it inactive as a repressor of the S phase and thereby promotes DNA replication (Mittnacht, 1998). Significantly, the pRB binding motif LXCXE (where X denotes any amino acid) is found in all known plant D cyclins. Moreover, LXCXE-dependent interactions between D cyclins from Arabidopsis and maize pRB proteins have been demonstrated in vitro and in a yeast two-hybrid assay (Ach et al., 1997; Huntley et al., 1998). The maize pRB proteins contain multiple putative CDK phosphorylation sites, and ZmRB-1 is efficiently phosphorylated in vitro by mammalian G1- and S-specific CDKs (Huntley et al., 1998). Moreover, maize pRB proteins are known to undergo changes in phosphorylation during the transition to endoreduplication in the endosperm (Grafi et al., 1996); however, phosphorylation by plant CDKs remains to be demonstrated.

Further evidence in support of a functional role for pRB proteins in plants comes from experiments showing that the overproduction of a maize pRB-like protein inhibits geminivirus replication. This suggests that pRB-like proteins in plants may also act as negative regulators of DNA synthesis (Xie et al., 1996). The observation that a pRB protein in maize leaves is highly produced in differentiating but not in proliferating cells (Huntley et al., 1998) suggests that some plant pRB-like proteins may be involved in the suppression of cell division during differentiation.

In mammals, hyperphosphorylated pRB disengages from inhibitory complexes with proteins such as E2F and MCM7 that are involved in the activation of S phase–specific transcription (Helin, 1998) and initiation of DNA replication (Leatherwood, 1998). In this regard, the *PROLIFERA* gene, which is required for megagametophyte and embryo development in Arabidopsis, encodes a protein that is 50% identical to the mammalian MCM7 proteins (Springer et al., 1995); it will thus be important to see whether PROLIFERA interacts with pRB proteins in plants.

Similarly, a putative homolog of mammalian E2Fs has recently been identified in wheat (Gutierrez, 1998), and there is an indication that, as is the case in animals, an E2F-like protein might also be a substrate for S phase–specific CDKs in maize endosperm (Grafi and Larkins, 1995). These observations, again circumstantial, imply that the control of the S phase in plants is more similar to that occurring in animal cells than in yeast cells.

PERSPECTIVES

Our understanding of the basic mechanisms that regulate cell division in plants has advanced considerably in recent years. Numerous key players have been identified, and an emerging model that integrates current knowledge is shown in Figure 2. Although initial investigations of the plant cell cycle appeared to be merely confirmatory, the field is now approaching a degree of maturity such that questions specific to plants may be addressed. Given the considerable differences between plants and animals in life strategies, we can expect numerous exciting breakthroughs in the near future. Further progress will depend on gaining a better understanding of the specific roles of the CDK/cyclin complexes. We need to find out which CDK/cyclin combinations are active over the course of the cell cycle and what their targets are. Furthermore, insights into the mechanisms of activation/deactivation should be gained and upstream regulators identified.

These formidable tasks will require considerable efforts in biochemistry and cell biology, efforts that will certainly pay off in the long run. Indeed, a thorough understanding of the operation of the basic cell cycle machinery promises to provide the information and tools necessary to understand how intrinsic developmental programs and environmental cues impinge on cell division. Early payoffs are already emerging as links have been found between auxin signaling and genes known to be involved in cell cycle–related protein degradation (Leyser et al., 1993; del Pozo et al., 1998; Ruegger et al., 1998) and between the action of cytokinins and the regulation of CDK activity (Zhang et al., 1996). We can look forward to understanding how cell division is initiated, how endoreduplication is regulated, and how cells exit the cell cycle to differentiate.

The cell cycle toolbox will also allow us to address fundamental questions with regard to the role of cell division in plant growth and architecture. For example, is cell division informed by growth, or is cell division the driving force for growth? This hotly debated subject pursued for quite some time by plant biologists has fueled arguments denying any role for the control of cell cycle in plant development and reducing cell division to the surveillance of cell growth (Clark and Schiefelbein, 1997).

Current research continues to fuel this debate. For example, in stressed cells of intercalary meristems of rice and of wheat leaves, modulations of the cell cycle have recently been shown to precede any detectable changes in cell growth (Lorbiecke and Sauter, 1998; Schuppler et al., 1998). These findings provide additional evidence that cell growth in plants is not the only driving force for cell division. Further progress in cell cycle research holds the promise of not only bringing a deeper understanding of how, when, and why plant cells divide but also of how cell division in plants might be modulated.

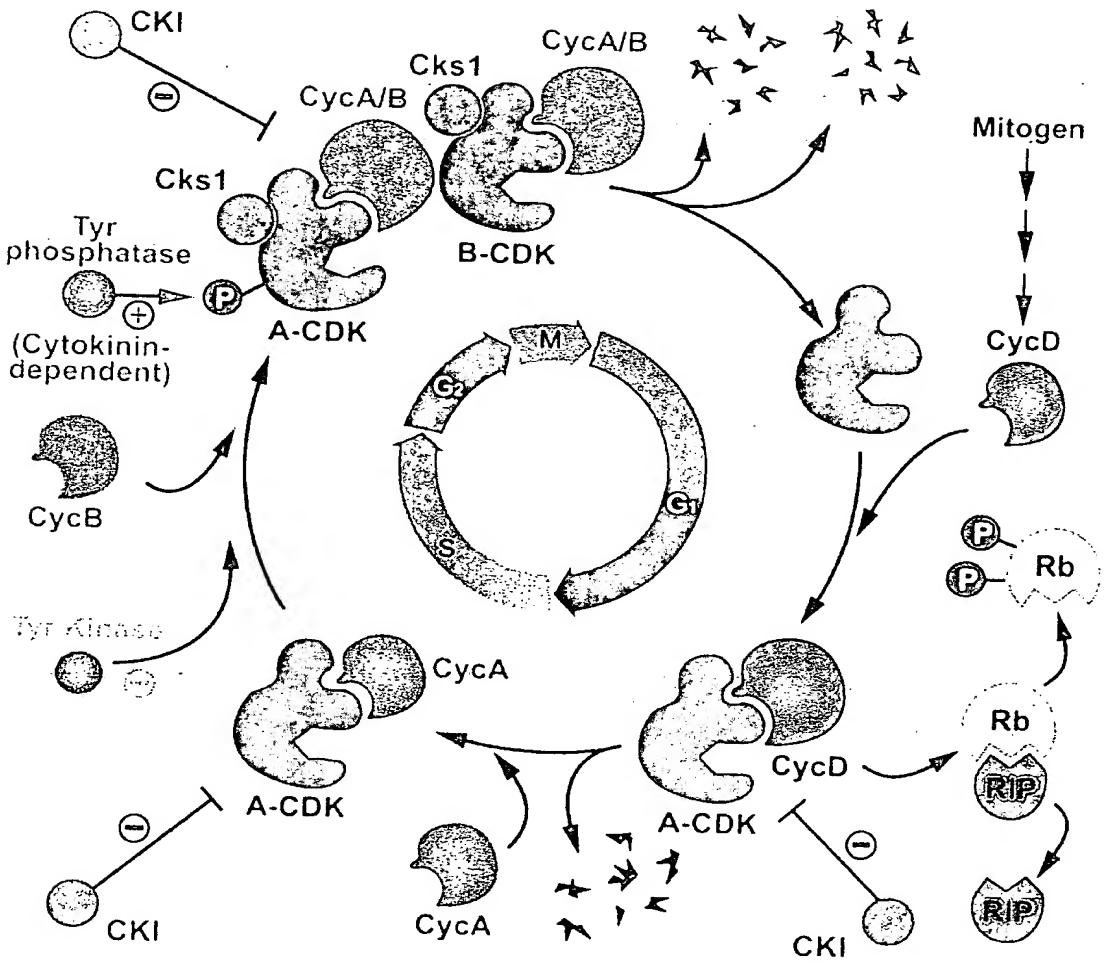

Figure 2. Model of Cell Cycle Control in Plants.

In consideration of the available data on plant cell cycle proteins and genes and in light of data on the corresponding gene products from heterologous systems, a model for cell cycle regulation in plants is proposed. Upon mitogenic stimulation (top right), D-type cyclins (CycD) are produced and associate with the A-type CDKs (A-CDKs). The resulting A-type CDK/cyclin D complexes phosphorylate a retinoblastoma-like protein (pRb), resulting in the release of pRB-interacting proteins (RIP) that in turn trigger the onset of S phase. The presence of PEST degradation sequences accounts for the short life span of the D-type cyclins. During the S phase, A-type cyclins (CycA) are synthesized to activate A-CDKs. As cells reach the end of the S phase, CDK activity is inhibited by Tyr phosphorylation. At G2, B-type cyclins appear. Because both A-type CDKs and B-type CDKs (B-CDK) display kinase activity at the G2/M transition point, both types of CDKs might coincidentally be present to associate with B-type cyclins. The correct functioning and activation of the mitotic CDKs require association with CKS1 docking factors (Cks1) and removal of the inhibitory phosphate group. The latter process was demonstrated to be cytokinin dependent. Specific degradation motifs in their protein sequences suggest that A- and B-type cyclins are destroyed during M phase, as indicated by colored fragments. The cell cycle could be arrested by the association of the A-type CDKs with CKIs.

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When plant cells decide to divide

Hilde Stals and Dirk Inzé

Progression through the cell cycle is central to cell proliferation and fundamental to the growth and development of all multicellular organisms, including higher plants. The periodic activation of complexes containing cyclins and cyclin-dependent kinases mediates the temporal regulation of the cell-cycle transitions. Here, we highlight recent advances in the molecular controls of the cell cycle in plant cells, with special emphasis on how hormonal signals can modulate the regulation of cyclin-dependent kinases.

The cell cycle is a highly ordered process that results in the formation of two daughter cells and is usually divided into four phases: G1, S (DNA replication), G2 and M (karyo- and cytokinesis) (Fig. 1). Ensuring that each new daughter cell receives a full complement of the hereditary material requires the correct alternation between S phase and M phase. The basic control mechanisms that regulate the progression through the cell cycle are remarkably well conserved through evolution. The main drivers of the cell cycle in yeast, mammals and plants are a class of highly conserved serine/threonine kinases known as the cyclin-dependent kinases (CDKs). Multiple mechanisms have evolved that strictly regulate the CDK activity to maintain the correct temporal ordering of critical cell cycle events, such as DNA replication and spindle assembly.

Here, we review recent advances in our understanding of how the cell-cycle control mechanisms act at the G1–S and G2–M transitions in plants, and we further highlight how hormonal signals are integrated into the cell cycle.

G1 entry and the G1–S transition

At a certain point in the G1 phase, known as START in yeast and as the restriction point in mammals, cells either continue through the cell cycle or stop to differentiate (Fig. 1). In mammals, progression through the restriction point is mediated by D-type cyclins, which also integrate extracellular signals. The continuous presence of growth factors induces the transcription of D-type cyclins and their association with CDK4 or CDK6. The CDK4/6–cyclin-D complexes that are activated via phosphorylation by a CDK-activating kinase (CAK) phosphorylate and inactivate the retinoblastoma protein (RB), thereby activating E2F-controlled genes, which are required for S phase progression. Most of the key players in the RB pathway are conserved throughout the evolution of multicellular organisms, including plants[1], in contrast with yeast and other unicellular organisms, in which no functional homologues of RB have been found to date.

Four classes of D-type cyclins have been identified in plants. Cyclins of the *CycD3* class play a role during S phase entry in response to plant hormones such as cytokinins and brassinosteroids, whereas *CycD2* and *CycD4* are activated earlier in G1 and respond to sugar availability[2–5]. In spite of the extensive list of plant CDKs, no direct equivalents of CDK4/6, the catalytic partners of D-type cyclins in animals, are known in plants. Based on sequence analysis with homologues from other eukaryotes, the family of plant CDKs is divided into five subtypes (A–E)[6]. In G1 phase, only CDKA;1, the homologue of mammalian CDK1, is produced and has been shown to interact with CycA2;1, CycD2;1 and CycD3;1 (Refs 7–10). Moreover, the CDKA;1–CycD3;1 complex of tobacco formed in insect cells can phosphorylate the tobacco RB-related protein *in vitro*[9]. RB-related proteins have been found in plant species such as maize, tobacco, *Arabidopsis*, pea, poplar and *Chenopodium rubrum*[11]. The maize RB protein, Zeama;RB1, and the human RB protein bind all classes of plant D-type cyclins *in vitro*, with the involvement of a conserved N-terminal LxCxE RB-interaction motif[12].

To date, protein inhibitors that modulate the CDK activity have only been identified in *Arabidopsis*, which contains genes for seven CDK inhibitors (ICKs) with distant sequence homology at their C-termini to the CDK-binding or inhibitory domain of $p27^{KIP2}$ (Refs 13,14; L. De Veylder, pers. commun.). The CDKA;1 kinase activity of *Arabidopsis* is inhibited *in vitro* by ICK1 and ICK2, both of which interact with CycD3;1 and CDKA;1 in an *in vitro* binding assay[13,14]. Transgenic *Arabidopsis* plants that overproduce ICK1 and ICK2 have a reduced CDK activity and fewer, but greatly enlarged, cells, showing for the first time the *in vivo* function of a CDK inhibitor *in planta*[15] (L. De Veylder, pers. commun.).

The RB tumour suppressor protein exerts its activity largely by binding the E2F family of DNA-binding transcription factors[16]. E2F sites are found in promoters of multiple plant and animal genes that are involved in cell-cycle progression and DNA replication[17–19]. E2F binds DNA as a heterodimer composed of two structurally related subunits, E2F and its heterodimerization partner (DP) (Fig. 1). The ability of an RB-related protein from maize to bind human and *Drosophila* E2F, and to inhibit the transcriptional activation of human E2F supported the existence of an RB–E2F pathway in plants[12]. The subsequent isolation of E2F homologues from wheat, tobacco, carrot and *Arabidopsis* confirmed this prediction[20–23]. Plant E2Fs exhibit transactivation properties in mammalian and plant cells, and have been shown to interact specifically with the E2F DNA-binding sequences[17,20,24]. Both the DNA-binding and transactivation activity of plant E2Fs required heterodimerization with a human DP protein, which suggested the existence of DP-related proteins in

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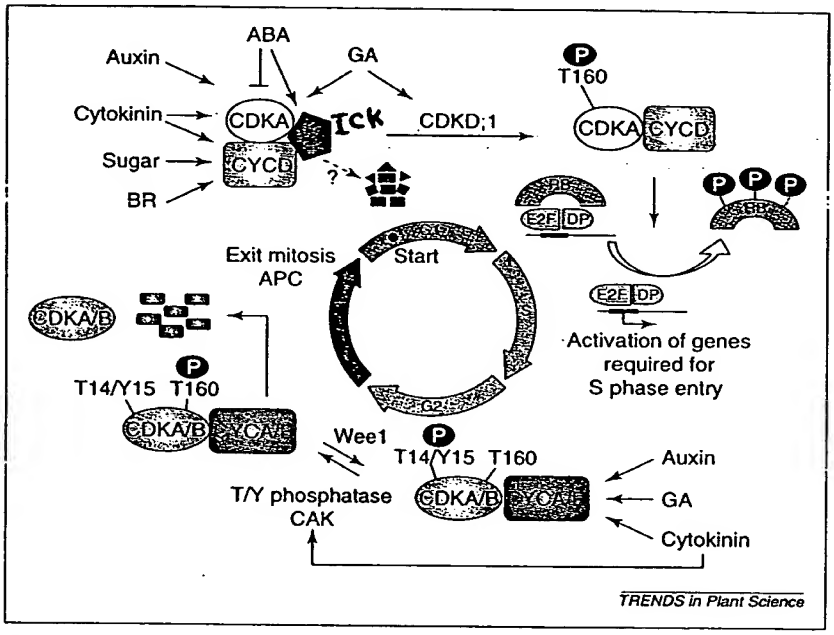

Fig. 1. Model for G1–S and G2–M transitions in plants based on results obtained in plants and on parallels with the mammalian cell-cycle control. During G1, several growth factors, such as auxin, cytokinin, abscisic acid (ABA), gibberellin (GA), brassinosteroids (BR) and sugar regulate the expression of D-type cyclins (CycD) and their catalytic subunit, cyclin-dependent kinase A (CDKA). Activation of the CDKA–CycD complex requires dissociation of the CDK inhibitory protein (ICK), the transcription of which is induced by the stress-responsive hormone ABA and phosphorylation of the Thr160 residue of CDKA by the CDK-activating kinase, CDKD;1, which is upregulated by GA. The active CDKA–CycD complex initiates the phosphorylation of retinoblastoma protein (RB) during late G1 phase, thereby releasing the E2F–DP complex that promotes the transcription necessary for progression into S phase. As mitotic activators auxin, cytokinin and GA also regulate the kinase activity of A- and B-type CDKs by activating the transcription of CDKs and of A- and B-type cyclins. The G2–M transition is associated with an activating Thr160 phosphorylation of CDK by a CDK-activating kinase (CAK) and by dephosphorylation of the inhibitory Tyr phosphorylation that is induced by cytokinin. A ubiquitin-dependent degradation pathway targets B-type cyclins for proteolysis by the anaphase-promoting complex (APC) at the metaphase–anaphase transition, thereby activating the exit from mitosis.

plants as well[20]. This hypothesis was confirmed by the isolation of two distinct *DP*-related genes from wheat and *Arabidopsis*[21,24]. *In vitro* binding assays demonstrated that the formation of E2F–DP complexes in *Arabidopsis* depends on the presence of their heterodimerization domains[21]. The cell-cycle-dependent expression of the plant *E2F* and *DP*-related genes, which are most abundant during early S phase[20–23], further supports their involvement in the regulation of S phase progression in plants.

All these findings strengthen the hypothesis that, during evolution, multicellular organisms as different as plants and animals evolved a similar pathway to control the G1–S transition. This might have originated in a primitive multicellular eukaryote before the divergence of the plant and animal kingdoms. Moreover, this common pathway relies on homologous proteins that are unrelated to those that control the G1–S transition in unicellular organisms such as yeast. In animals, the RB pathway is involved not only in cell-cycle progression but also in the control of programmed cell death. Whether this is also the case for plants is still unknown. Nevertheless, the recent discovery of plant genes encoding prohibitins[25] suggests that there might be a connection between cell-cycle control and cell death in plants as well. Homologues of other animal tumour suppressor genes, such as *p53*, have not been found in plants yet[26].

G2–M transition

Once the cell has duplicated its DNA during the S phase, its next tasks are to generate a mitotic spindle, disassemble the nuclear envelope, condense its chromosomes and align each pair of sister chromatids on the metaphase plate. Expression analysis of five different classes of plant CDKs revealed that the A-type CDKs, like the animal and yeast homologues, are constitutively transcribed. By contrast, the B-type CDKs, which represent a plant-specific gene family, show a cell-cycle-dependent expression pattern, with transcript and protein levels accumulating in G2–M cells[27]. Immunoprecipitation with specific antibodies against A- and B-type CDKs clearly showed histone-H1-phosphorylating activity for CDKA in S, G2 and M phases, and for CDKB during G2–M transition[28–30] (A. Porceddu *et al.*, unpublished). These data suggest that, in contrast with animals and yeast, at least two kinases regulate the G2–M transition in plants (Fig. 1). A potential role for B-type CDKs during entry of mitosis is further supported by the observation that downregulation of B-type CDKs in transgenic plants increases the relative duration of G2 phase (A. Porceddu *et al.*, unpublished).

Limited data are available on the cyclin partners of CDKA or CDKB during the G2–M transition, although both proteins probably bind plant cyclins expressed at the same timepoint[27]. The number of known plant cyclin genes has increased rapidly during the past decade. Completion of the genome-sequencing program of *Arabidopsis* indicated the existence of 27 different cyclins that can be classified as A, B, D and H types by sequence comparison with their mammalian homologues (K. Vandepoele, pers. commun.): the A, B and D types are studied most intensively[27]. The transcript levels of most of the plant D-type cyclins are almost constant during the cell cycle, reminiscent of the animal D-type cyclins[31]. However, two genes for cyclin D homologues from tobacco (*Nicta;CycD2;1* and *Nicta;CycD3;1*) show a mitotic accumulation of their transcripts in synchronized BY-2 cells[32], suggesting that these D-type cyclins are required for entry into or progression through mitosis. Alternatively, the mitotic accumulation of the D-type cyclins could be a BY-2-cell-specific phenomenon as the result of a deregulation of their expression caused by long-term culturing.

Most plant A- and B-type cyclins show a mitotic expression pattern[27], with the exception of *Medsa;CycA2;1* from alfalfa. This cyclin has constitutive transcript and protein levels during the cell cycle, yet its associated kinase activity is biphasic, peaking during S phase and at the G2–M transition[10]. The CDK responsible for both histone-H1-kinase activities is probably CDKA;1, because CycA2;1 interacts with CDKA;1 in a yeast two-hybrid system[10]. The kinase activity associated with B-type

cyclins correlates well with their protein levels, being highest during the G2–M phase transition and disappearing at the exit of mitosis[33]. Similar to their animal and yeast homologues, plant mitotic cyclins contain a destruction box, which targets them for ubiquitin-dependent degradation during mitosis[33,34] (Fig. 1). It has been well established in other systems that CDK activity needs to be switched off during mitotic exit and during G1 to continue through the cell cycle. Overproduction of a non-degradable B-type cyclin in yeast[35] and mammalian systems[36] causes a mitotic arrest. However, no mitotic arrest was observed[37] during the ectopic expression of a non-degradable *clb2* in yeast at modest levels. Similarly, ectopically expressed *Nicta;CycB1;1* in tobacco BY-2 cells did not arrest the cell cycle, suggesting that plants, like yeast, might possess additional mechanisms to inactivate the CDK activity[33].

Full activation of the mitotic CDK activity requires not only cyclin association but also specific phosphorylation–dephosphorylation events (Fig. 1). Dephosphorylation at the inhibitory phosphorylation sites (Thr14 and Tyr15 in human CDK2) by a dual-specificity phosphatase, CDC25, and phosphorylation of a Thr residue within the T-loop region (Thr160 in human CDK2) are additional regulatory mechanisms[38]. Phosphorylation of the Thr160 residue is catalysed by at least two structurally distinct types of CAKs: the trimeric CDK7–CycH–Mat1 complex in metazoans and the single-subunit Cak1 in budding yeast[39]. Fission yeast has both CAK types, with the multisubunit kinase (Mcs6) acting as the Cdc2-activating kinase and the single-subunit kinase (Csk1) as the Mcs6-activating kinase[40].

To date, two plant CAKs have been isolated from rice and *Arabidopsis*, and they have been renamed as CDKD;1 (Ref. 6). *In vitro*, rice CDKD;1 not only phosphorylates human CDK2 and rice CDKA;1 at the Thr residue within their T-loop but also, similarly to the CDK7–CycH–Mat1 complex, phosphorylates the C-terminal domain (CTD) of RNA polymerase II of *Arabidopsis*[41]. Moreover, the rice CDKD;1 kinase is regulated positively by an H-type cyclin, Orysa;CycH;1 (Ref. 42). However, because the transcription of both genes is induced when cells enter S phase, the rice CAK is probably more involved in regulating S phase progression[42,43]. The *CAK1At* of *Arabidopsis*, now designated *Arath;CDKD;1* (Ref. 6), was isolated as a suppressor of the temperature-sensitive *cak* mutant of budding yeast[44]. Although *Arath;CDKD;1* has the closest similarity to *CDK7* from metazoans, a phylogenetic analysis showed that *Arath;CDKD;1* is distinct from *CDK7* and also unrelated to *Cak1* of *Saccharomyces cerevisiae*, suggesting that it might be a novel type of CDK-activating kinase[44]. Moreover, *Arath;CDKD;1* can phosphorylate only human CDK2 and not *Arabidopsis* CTD (Ref. 44); this contrasts with the CAKs of vertebrates and fission yeast, which phosphorylate both CTD and CDKs.

Ectopic expression of *Arath;CDKD;1* in *Arabidopsis* caused an extensive phosphorylation of CDKA;1. Surprisingly, the associated kinase activity of CDKA;1 was reduced without effecting the protein amount[45], indicating that other limiting mechanisms are involved. Thus, plants seem to have two distantly related CAKs (Ref. 6), which suggests a redundancy for the phosphorylation of CDKs. However, these plant CAKs might have distinct functions, acting at different phases of the cell cycle and/or phosphorylating different subtypes of CDKs. An unanswered, but interesting, question is whether CAK of *Arabidopsis* interacts with a cyclin subunit to form an active CAK complex. Identification of the regulatory subunit might help determine the substrate specificity of both plant CAKs. Recently, a putative H-type cyclin has been identified in the *Arabidopsis* genome (K. Vandepoele, pers. commun.).

A WEE1 homologue, which is responsible for the phosphorylation of CDKs at Thr14/Tyr15, has been isolated from maize endosperm and inhibited the $p13^{Suc1}$-adsorbed mitotic CDK activity[46]. In maize endosperm, the M-phase-associated CDK activity decreases at the onset of endoreduplication because of the presence of an inhibitory factor, whereas the S-phase-related kinases are induced[47]. Because *Zeama;WEE1* transcripts are most abundant in actively dividing tissue and accumulate in maize endosperm during the period of endoreduplication[46], this kinase could be one of the factors that inhibit the mitotic CDK activity.

The CDC25 homologue of plants has not been cloned yet and the finalization of the *Arabidopsis* genome-sequencing program showed that *Arabidopsis* does not have a CDC25 homologue[26]. Nevertheless, recombinant CDC25 phosphatase from yeast or *Drosophila* activates the $p13^{Suc1}$-bound CDK fraction of tobacco and alfalfa cells *in vitro*[30,48]. The importance of the Thr/Tyr phosphorylation for the timing of mitosis entry in plant cells is further supported by tobacco transformants that express the yeast *CDC25* gene, in which cells divide at a reduced cell size[49]. Therefore, an unidentified dual-specificity phosphatase could be responsible for the dephosphorylation of the inhibitory Thr/Tyr residues in plants.

Hormone signalling and cell division

Owing to their sessile lifestyle, plants have to respond to local environmental conditions by changing their physiology and redirecting their growth. Signals from the environment include light and pathogen attack, temperature, water, nutrients, touch, and gravity. In addition to local cellular responses, some stimuli are communicated across the plant body by hormones, which consequently play an important role in diverse aspects of plant growth and development. At a cellular level, auxin affects division, expansion and differentiation. Auxin increases both CDKA;1 and mitotic cyclin mRNA levels in roots in conjugation with the induction of cell division[50–52] (Fig. 1).

Although auxin is sufficient to induce the *CDKA;1* expression, it is not in itself enough to stimulate cell division in most cultured cells[48,53]. In tobacco root explants, the catalytic activity of the kinase and the following entry into mitosis is induced only by the addition of cytokinin, stimulating Tyr dephosphorylation of CDKA;1 kinase[48].

Recent studies with *Arabidopsis* have revealed that the ubiquitin proteolysis system plays a central role in the auxin response pathway[54]. The *auxin transport inhibitor resistant 1* (*TIR1*) gene encodes an F-box protein that interacts with plant orthologues of SKP1 (called ASK1 and AKS2) and a cullin protein (Arath;CUL1) to form the ubiquitin protein ligase complex SCF (Ref. 54). How does auxin affect the cell-cycle machinery? One of the effects of auxin is to induce lateral root formation by initiating pericycle cell division from G2 phase, a process that is preceded by increased *CycB1;1* expression. In *tir1-1* mutants (which carry an *Arath;CycB1;1–gus* reporter gene) grown on unsupplemented nutrient medium, reporter-gene expression is lower than that of wild-type seedlings, corresponding to the reduced number of lateral roots that develops in *tir1-1* mutants. These observations indicate that *TIR1* is required directly or indirectly before the expression of *Arath;CycB1;1* in lateral root development[54]. Thus, auxin-promoted pericycle cell division might be achieved by SCF^{TIR1}-facilitated degradation of one or more negative regulators of the cell cycle.

Cytokinins are necessary, in concert with auxin, for cell division at the G1–S and G2–M transitions in a variety of cultured plant cells and *in planta*. When exogenous cytokinin is applied, the expression of *CDKA;1* increases and its kinase activity is induced by promoting the CDKA;1 inhibitory Tyr dephosphorylation at the G2–M transition[48] (Fig. 1). At the G1–S transition, cytokinin regulates cell-cycle progression partly by inducing *CycD3;1* transcription[31] (Fig. 1). Constitutive expression of *CycD3;1* in *Arabidopsis* is sufficient to bypass the need for cytokinins in tissue culture, indicating that cytokinin might promote cell proliferation by inducing this D-type cyclin[4]. *CycD3* transcripts are also upregulated by brassinosteroids (BRs) (Fig. 1), which can substitute for cytokinin in promoting cell proliferation of callus and suspension cultures[3]. Unlike the cytokinin-induced *CycD3* transcription, the BR signal is not mediated by protein phosphorylation but needs protein synthesis[3]. *In planta*, BRs regulate hypocotyl elongation. BR treatment can partly rescue the short-hypocotyl phenotype of the *CDKB1;1*-inducible antisense *Arabidopsis* seedlings when grown in the dark[55]. Furthermore, BRs enhance the *CDKB1;1* expression observed in the hook region of dark-grown seedlings[55]. These results indicate that the *CDKB1;1* gene induced by BRs in darkness could titrate out the *CDKB1;1* antisense mRNA in the inducible antisense transgenic seedlings. However, because BRs cannot fully rescue the short-hypocotyl phenotype of induced antisense *CDKB1;1* transgenics, it is possible that BRs are only part of the regulatory system controlling *CDKB1;1* expression[55].

Upon submergence in water, cell division and cell elongation are accelerated in the intercalary meristems of deep-water rice internodes by gibberellin. This mitogenic hormone initially induces, by an unknown mechanism, A-type CDK and rice CDKD;1 mRNAs at the G1–S transition[43] (Fig. 1). Before the subsequent increase in mitotic B-type cyclin gene expression during late G2, *CycA1;1* and *CDKB1;1* are induced[56] (Fig. 1), which suggests that they might play distinct roles in the regulation of the G2–M transition.

The stress-responsive hormone abscisic acid inhibits cell division in response to adverse environmental cues. This effect might be mediated by the induction of a CDK inhibitor, *ICK1*, which might, together with decreased *CDKA;1* gene expression, result in the observed lower CDK activity[14,52] (Fig. 1).

The receptor mechanism that senses and transmits growth factors in plants is largely unknown. Receptor tyrosine kinases, which play a central role in binding growth factors and thereby regulate several cytoplasmic signal transduction cascades in mammalian cells, do not seem to exist in plants[26]. However, structurally similar receptor kinases that phosphorylate Ser/Thr residues could be their functional equivalents. The best-characterized signa transduction cascade is the mitogen-activated-protei

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Questions for future research

In recent years, considerable progress has been made in understanding the core cell-cycle machinery. The major challenge for future research is to elucidate the mechanisms by which developmental signals and environmental cues communicate with the basic cell-cycle engine.

- What triggers cell division?
- How do plant hormones communicate with the core cell-cycle machinery?
- What causes dividing cells to exit the cell cycle, to elongate and differentiate?
- Which signals convert a mitotic cell cycle into an endoreduplicating cell cycle?
- Which mechanisms are involved in the positioning of the division plane?
- How are cell division and cell growth coupled?

Answering these questions will require a worldwide, multidisciplinary effort. Much progress should be made by combining knowledge on cell-cycle genes (such as cell-cycle phase and cellular specificity of expression, subcellular localization of the proteins, and two-hybrid interactors) with knowledge on signalling pathways that are important for development and interaction with the environment. Two model systems will probably drive future progress: tobacco BY-2 cells, to study the cellular signalling, and *Arabidopsis*, to integrate cell cycle and development. The availability of ever-growing collections of *Arabidopsis* mutants will be of invaluable help. In addition, new collections of conditional mutants, such as temperature-sensitive mutants for growth, will probably contribute to the further elucidation of cell division as an essential process.

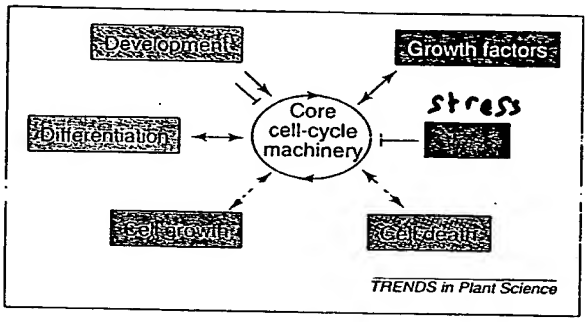

Fig. 2. The major challenge for the future will be the study of the clusters of different signal transduction pathways in plants, by which different processes, such as stress responses, development, cell elongation and differentiation, hormone responses, and cell death are integrated into the core of the cell-cycle machinery and thereby modulate patterns or rate of cell proliferation. Bars indicate inhibition; single-headed arrows indicate activation; double-headed arrows indicate unknown regulatory mechanism.

kinase (MAPK) cascade, which is implicated in cellular activities including proliferation, differentiation, division and death[57]. Although various components of the MAPK cascade have been isolated in plants, no MAPK signal transduction pathway that integrates mitotic stimuli into the core of the plant cell cycle has been established conclusively.

Conclusions

Future challenges lie in unraveling the clusters of different signal transduction pathways in plants by which environmental cues are integrated into the control of the cell cycle, thereby changing the patterns or rate of development. Because the cell cycle is intimately linked to plant development, engineering cell-cycle genes has proved to be a powerful tool to affect plant architecture and important agricultural traits, such as growth rate[58]. Now that the genome sequence of *Arabidopsis* has been finalized and those of other plants are being sequenced, functional genomics and proteomics will be important techniques for determining the global molecular responses that impinge on the cell cycle (Fig. 2). This immense task should ultimately enable us to understand how the core of the cell-cycle machinery is integrated with processes as different as stress responses, development, cell elongation and differentiation, hormone responses and cell death (Fig. 2).

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Functional genomics of plant photosynthesis in the fast lane using *Chlamydomonas reinhardtii*

Rachel M. Dent, Miehie Han, and Krishna K. Niyogi

Oxygenic photosynthesis by algae and plants supports much of life on Earth. Several model organisms are used to study this vital process, but the unicellular green alga *Chlamydomonas reinhardtii* offers significant advantages for the genetic dissection of photosynthesis. Recent experiments with *Chlamydomonas* have substantially advanced our understanding of several aspects of photosynthesis, including chloroplast biogenesis, structure–function relationships in photosynthetic complexes, and environmental regulation. *Chlamydomonas* is therefore the organism of choice for elucidating detailed functions of the hundreds of genes involved in plant photosynthesis.

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With the genome sequence of the first photosynthetic eukaryote fully characterized, the path appears set for *Arabidopsis* to dominate the field of plant biology in the next decade. But *Arabidopsis* is not the ideal organism for all fields of plant research, and photosynthesis is one area where other models have major advantages (Table 1). *Chlamydomonas reinhardtii* (Fig. 1), for example, has been used as a model organism in photosynthesis research for >40 years, and the use of this unicellular green alga in biochemical, biophysical and genomic approaches, to the study of photosynthesis and photoprotection has been reviewed by several authors recently[1–7]. This review describes several examples of how molecular genetic studies of *Chlamydomonas* have provided new insights into photosynthesis. We will highlight the comparative merits of *Chlamydomonas* as a model photosynthetic organism and discuss how it can make future contributions to the functional genomics of photosynthesis.

Advantages of *Chlamydomonas* for studying photosynthesis

Chlamydomonas has several attributes that make it an excellent organism for basic genetic studies of plant photosynthesis (Table 1). Its photosynthetic apparatus is closely related to that of vascular plants, and it is also a eukaryote, with photosynthesis genes encoded by both the nuclear and chloroplast genomes. As a unicellular organism, *Chlamydomonas* has the advantages of a microbial lifestyle without the complications of multicellularity. Synchronous or asynchronous cultures of *Chlamydomonas* grow quickly with a doubling time of less than ten hours, and the cells behave homogeneously in terms of physiological and biochemical characteristics. Because *Chlamydomonas* is haploid and has a controlled sexual cycle with the possibility of tetrad analysis (Fig. 2), it is an excellent genetic model.

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Effect of Water Stress on Cell Division and Cell-Division-Cycle 2-Like Cell-Cycle Kinase Activity in Wheat Leaves[1]

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In wheat (*Triticum aestivum*) seedlings subjected to a mild water stress (water potential of −0.3 MPa), the leaf-elongation rate was reduced by one-half and the mitotic activity of mesophyll cells was reduced to 42% of well-watered controls within 1 d. There was also a reduction in the length of the zone of mesophyll cell division to within 4 mm from the base compared with 8 mm in control leaves. However, the period of division continued longer in the stressed than in the control leaves, and the final cell number in the stressed leaves reached 85% of controls. Cyclin-dependent protein kinase enzymes that are required in vivo for DNA replication and mitosis were recovered from the meristematic zone of leaves by affinity for $p13^{suc1}$. Water stress caused a reduction in H1 histone kinase activity to one-half of the control level, although amounts of the enzyme were unaffected. Reduced activity was correlated with an increased proportion of the 34-kD Cdc2-like kinase (an enzyme sharing with the Cdc2 protein of other eukaryotes the same size, antigenic sites, affinity for $p13^{suc1}$, and H1 histone kinase catalytic activity) deactivated by tyrosine phosphorylation. Deactivation to 50% occurred within 3 h of stress imposition in cells at the base of the meristematic zone and was therefore too fast to be explained by a reduction in the rate at which cells reached mitosis because of slowing of growth; rather, stress must have acted more immediately on the enzyme. The operation of controls slowing the exit from the G1 and G2 phases is discussed. We suggest that a water-stress signal acts on Cdc2 kinase by increasing phosphorylation of tyrosine, causing a shift to the inhibited form and slowing cell production.

A decrease in soil water potential due to drought or salinity decreases the rate of leaf expansion, whereas root expansion is much less affected (Munns and Sharp, 1993). If the root water potential decreases suddenly, the response of leaf expansion is so rapid and large (Cramer and Bowman, 1991) that it must be due to a change in the rate of expansion of existing cells, rather than to a change in the rate of production of new cells. However, when plants have grown for some time in soils of low water potential, smaller leaves with fewer cells are formed (Clough and Milthorpe, 1975; Randall and Sinclair, 1988; Lecoeur et al., 1995). These observations suggest that reduced cell formation during water stress may limit final leaf size.

Little is known about the effects of water stress on rates of cell division or on mitotic activity in leaves. Roots have received more attention. In roots there was a rapid decrease in mitotic activity after imposition of water stress (Yee and Rost, 1982; Robertson et al., 1990b; Bitonti et al., 1991; Bracale et al., 1997), and a similar response was found for soybean hypocotyls (Edelman and Loy, 1987). These sudden decreases in mitotic activity suggest, but do not prove, that water stress caused a sudden decrease in the rate at which new cells were being produced. To our knowledge, the only study to measure rates of cell production under water stress was done by Sacks et al. (1997), who found a 40% reduction in the rate of cell division in cortical cells of the primary root of water-stressed maize.

Evidence that the extent of cell production may limit growth has come from the stimulation of root growth that follows increased expression of a cyclin cell-cycle gene in Arabidopsis (Doener et al., 1996). The product of the cdc2 (*cell-division-cycle*) gene, the protein kinase $p34^{cdc2}$ (Nurse, 1990), here referred to as Cdc2 kinase, plays a major role in driving the cell cycle. Genetic analysis in *Schizosaccharomyces pombe* has shown that cdc2 function is required at the start of the S phase and at entry into mitosis (Nurse and Bissett, 1981). The Cdc2 kinase is the first discovered member of a group of CDKs found in higher eukaryotes that require association with a cyclin protein to become enzymically active (Norbury and Nurse, 1992).

At mitosis the Cdc2 kinase is the dominant active CDK; at this time its activity is severalfold higher than at other times in the cell cycle (Tsai et al., 1991; for review, see Stern and Nurse, 1996). This drives the structural changes of nuclear envelope disassembly, chromosome condensation, and mitotic spindle assembly in yeasts and animals (for review, see Nurse, 1990; Nigg, 1995) and in plants (Hush et al., 1996). The importance of the Cdc2 kinase in cell division in plants was indicated by the ability of plant cdc2 genes to support division in yeasts (Hirt et al., 1991) or, in the negative mutant form, to block division in plants (Hemerly et al., 1995). The importance of Cdc2 is further indicated by the positive correlation of cdc2 gene expression

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Abbreviations: CDK, cyclin-dependent protein kinase enzyme; $p13^{suc1}$, 13-kD universal mitotic protein with affinity for Cdc2 binding in vitro.

with regions of cell division (Gorst et al., 1991; John et al., 1991; Martinez et al., 1992; Hemerly et al., 1993; for review, see John, 1996). Furthermore, a role of mitotically active CDK in driving plant mitosis is directly shown by the stimulation of mitosis that is induced by injection of the mitotically active form of plant Cdc2 protein into stamen hairs of *Tradescantia virginiana* (Hush et al., 1996).

Activation of Cdc2 kinase at the initiation of mitosis requires the binding of mitotic cyclin and the phosphorylation of Thr and Tyr residues before catalytic activity is released by the removal of phosphate from Tyr 15 (and also from Thr 14 in animal cells) by Cdc25 phosphatase (Norbury and Nurse, 1992). As cells approach mitosis there is a decline in the abundance of inactive Tyr-phosphorylated enzyme and an increase in active Tyr-dephosphorylated enzyme (Russell and Nurse, 1986, 1987; Moreno et al., 1990; Lew and Kornbluth, 1996). The same decline in Tyr phosphorylation of Cdc2 kinase has recently been detected as the mechanism of cytokinin induction of plant mitosis (Zhang et al., 1996). This is an important element in regulating plant cell division and here we report its contribution to environmental responses.

To investigate possible environmental influence over cell division in the developing leaf, we subjected wheat (*Triticum aestivum*) seedlings to a mild (soil) water stress at a very early stage of development and recorded the spatial extent of mitotic activity throughout leaf development. Previous publications showed that only in leaves that were very small at the onset of a water stress (e.g. 2% of final leaf area; 5% of final cell number) was the number of cells produced per leaf affected by stress (Clough and Milthorpe, 1975; Randall and Sinclair, 1988; Lecoeur et al., 1995). Calculations from Foard and Haber (1961) indicated that less than 3% of the mesophyll cells in the first leaf of a wheat seedling are formed during seed development; therefore, we used germinating wheat seedlings maintained in the dark at high humidity to study the effects of a mild and steady water stress that would have an impact on the plant at an early stage of leaf development.

We measured the effect of stress on the spatial extent of cell division throughout leaf development to locate the tissue most responsive to stress. We focused on mesophyll cells rather than other cell types because mesophyll cells are the major cell type in leaves and make the greatest contribution to the volume of this tissue. Epidermal cells, which have been the subject of most studies of cell expansion or division, have a much shorter zone of division than do mesophyll cells (associated with their larger final dimensions), and preliminary observations showed that their mitotic activity was restricted to the basal few millimeters of the leaf and terminated sooner than that of mesophyll cells (data not shown). We then investigated whether mild water stress in the wheat leaf affects regulation of the cell cycle by testing for effects on cell-cycle progression and on Cdc2 kinase activity. We report evidence that water stress has rapid effects on Tyr phosphorylation and activity of a Cdc2-like enzyme and propose that these contribute to reduced cell-division activity.

MATERIALS AND METHODS

Growth Conditions

Wheat (*Triticum aestivum* cv Kite) seeds were soaked in water for 24 h in a Petri dish and then transferred to pots 10 cm wide and 25 cm deep containing vermiculite that was either fully or partially hydrated with water. Seeds were placed about 5 cm beneath the surface of the vermiculite. The level of hydration that reduced leaf elongation rate by about 50% was found to be 22% (grams of water per gram of vermiculite), which had a water potential of −0.3 MPa as determined with an isopiestic psychrometer (Boyer and Knipling, 1965). Seedlings were grown at 23°C in the dark in a humidified chamber. This method was chosen because the lack of transpiration meant that the soil water was not depleted and that the plant water potential would not change. Transplanting and nondestructive length measurements were done under a green safelight giving less than 0.1 μmol PAR m^{-2} s^{-1}.

Measurement of Blade-Elongation Rate

Lengths of leaf 1 were measured from leaf base to tip with a ruler every 24 h on 30 replicates, and the position of the ligule was recorded under a dissecting microscope. Sheath lengths were subtracted from the total leaf length, and blade lengths were recorded until the blade was fully elongated. Seed reserves were still adequate for growth at this stage but were depleted before the sheath finished growing. This experiment was carried out four times. In one experiment the second leaf was dissected and measured.

Measurement of Mitotic Activity

For microscopic analysis 10 leaves with lengths closest to the mean of 30 leaves were chosen. Because the stressed leaves were elongating at only about one-half the rate of the controls, samples from the stressed plants were taken every 2nd d after d 2. Samples were always taken at the same time of day. The basal 30 mm of the shoot was excised, fixed in formalin-acetic acid-70% ethanol (1:1:18), dehydrated in an ethanol-*t* butanol series under vacuum, and infiltrated with paraffin (Paraplast Plus, Sherwood Medical Laboratories, St. Louis, MO; melting point 56–57°C) according to the method of Volenec and Nelson (1981). Longitudinal 8-μm-thick sections were hydrolyzed with 1 N HCl for 10 min at 60°C, stained with Schiff's reagent for 16 h by the Feulgen method, and counterstained with Fast Green (CI 42053, Sigma; 0.01% in 95% ethanol for 30 s).

The mitotic index of the mesophyll cells of the first leaf was estimated by scoring the percentage of cells in the two cell layers beneath each epidermis that were clearly in metaphase, anaphase, or telophase. Prophase was not always distinctive, therefore, this phase was not scored. This was done for consecutive fields of view; for a given section, cells along the entire meristem were examined. In total, 32 files of cells were measured in eight sections from four

different leaf samples for each period. The position of the ligule was recorded. The data are presented as the distances from the base of the leaf or from the ligule, if present, in segments of 0.45 mm.

In harvesting plants at 24-h intervals, we assumed that there would be no synchrony of cell division, which is the usual situation for plants grown under constant conditions. This assumption was later confirmed by the absence of coordinated mitotic events observed across the tissue and by the constancy of CDK activity, which increases in the G2 phase (Zhang et al., 1996), but was found to have a similar activity in tissue harvested at many times during a 24-h time course (see "Results").

Estimation of Final Cell Number per Leaf

Final cell size was measured in leaf blades harvested at d 7 (unstressed) or d 12 (stressed) of treatment in two additional experiments replicated for this purpose. Measurements of cell length were made on the layer of mesophyll cells below the epidermis after clearing with methanol (10 min at 70°C) and lactic acid. Because mature mesophyll cells in wheat vary in length and shape with position in the leaf (Jung and Wernicke, 1990), leaves were divided into quarters along their length, and the average cell length for each quarter was estimated. Ten cells were measured in each quarter of nine leaves from each of the two experiments (a total of 90 cells for each part of the leaf per treatment). The total number of cells produced in a given file along the blade of the fully expanded leaf of stressed plants was calculated from the lengths of the mature leaf and cells.

Plant Material for Cell-Cycle Experiments

For most experiments plants were harvested 48 h after transfer to vermiculite. At this stage the first leaf of the unstressed plants averaged 17 mm in length, and that of the stressed plants averaged 8 mm. At this stage the second leaf was only 2 mm long in the unstressed plants and one-half of this in the stressed plants. The shoot was cut at its base from the seed coat, and 3-mm segments were dissected starting from the base, covering the meristem, and in some experiments extending beyond it. In most experiments only the segments 0 to 3 mm and 3 to 6 mm from the base were examined, because these contained the most actively dividing tissue. Segments were frozen immediately in liquid N_2 and stored at −80°C.

For the time-course experiment seeds were soaked for 24 h as usual and were then transplanted to pots containing fully hydrated vermiculite for another 36 h before a second transfer to pots containing either fully or partially hydrated vermiculite. The period of 36 h in well-watered conditions permitted the seedlings to grow sufficiently long to allow dissection into two 3-mm segments at the start of the stress treatment. This treatment is called the "transplantation protocol" to distinguish it from the procedure used in most of the experiments.

Biochemical Techniques

Purification of a Cdc2-like protein was carried out by grinding the leaf segments in liquid N_2 and mixing vigorously at 0°C with NDE buffer (20 mM Hepes, 50 mM β-glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA, 5 mM NaF, 2 mM DTT, 5 μM leupeptin, 5 μM pepstatin, 2 μg/mL aprotinin, 0.5 mM sodium orthovanadate, 10 μM ammonium molybdate, and 10 mg/mL sodium 4-nitrophenylphosphate, with PMSF added to 0.5 mM final concentration immediately before use), pH 7.4. Protein extracts were incubated for 2 h at 4°C with 40 μL of bead suspension (20-μL beads containing 8 mg p13/mL beads, which are hereafter referred to as p13 beads) to bind the Cdc2-like protein (John et al., 1991; Hepler et al., 1994).

The beads were washed twice at 0°C with 400 μL of 5 mM Na_2HPO_4, 4 mM NaH_2PO_4, 2 mM EDTA, 150 mM NaCl, 1% Nonidet P40, 5 μg/mL leupeptin, 0.1 mM sodium vanadate, and 50 mM NaF, pH 7.0, and then washed once with HBK (25 mM Hepes, 1 mM EGTA, 5 mM $MgCl_2$, 160 mM KCl, and 1 mM DTT). Cdc2-like protein was eluted from the beads by incubation for 10 min at 4°C with 45 μL of free $p13^{suc1}$ (0.5 mg/mL) in HBK, and the beads were discarded by centrifugation. The activity of the purified protein was measured immediately in a kinase assay. Linear recovery of enzyme was obtained from amounts of extract containing less than 350 μg of protein incubated with 20 μL of p13 beads.

The kinase assay was carried out on 20 μL of purified Cdc2-like protein in an incubation volume of 50 μL (John et al., 1991; Zhang et al., 1996), with the modification that 20 μL of the terminated assay was spotted onto Whatman P81 paper and washed five times for 3 min in 75 mM H_2PO_4, and the radioactivity was determined in a scintillation counter to quantify the amount of $[^{32}P]PO_4$ transferred to H1 histone.

To estimate the level of Cdc2-like protein relative to other proteins, total protein was extracted from leaf tissue, frozen, and ground in liquid N_2 into NDE buffer (John et al., 1990). Fifty-microgram samples of leaf protein were separated by SDS-PAGE on a 12% acrylamide gel. Proteins were transferred to nitrocellulose and probed with antibody against the 16-amino acid region EGVPSTAIREISLLKE, which is universally conserved in all known cdc2 molecules, as described previously (Lee and Nurse, 1987; John et al., 1989, 1990). Bound antibody was detected with ^{125}I-anti-rabbit IgG $F(ab')_2$ at 0.5 mCi/L (IM1340, Amersham) visualized by 72-h exposure to a phosphor-imaging screen. Samples to be compared were electrophoresed and transferred onto the same piece of nitrocellulose.

To measure the Tyr-phosphorylation state, the enzyme was purified with $p13^{suc1}$ beads from samples of extract containing 300 μg of protein, separated by 12% acrylamide SDS-PAGE, transferred to nitrocellulose, blocked with 1% BSA in buffered saline, and probed with anti-phospho-Tyr antibody (mouse monoclonal PY-20, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-mouse antibody (Chemicon, Temecula, CA) as described by Zhang et al. (1996). Bound antibody was detected with ^{125}I-anti-rabbit

IgG F(ab')$_2$ at 0.5 mCi/L (IM1340, Amersham) after 72 h of exposure to a phosphor-imaging screen.

Measurement of G1/G2 Frequency

The frequency of G1- and G2-phase nuclei was measured in longitudinal sections of leaves after 48 h of treatment, which were embedded in paraffin and stained by the Feulgen method. The cells measured were those in the two layers adjacent to both epidermes, i.e. the cells destined to become mesophyll cells. The intensity of nuclear staining was quantified using a Nikon Optiphot microscope with white light. Images obtained with a SIT television camera (Dage MTI, Michigan City, IN) were recorded in digitized form using an image-processing attachment (Image-1, Universal Imaging Corp., Chester, PA) and analyzed using imaging software (Image-1, version 4.0, Universal). A minimum area enclosing the whole nucleus was measured, and for each cell a background intensity was estimated from the average of three areas of the same size in the adjacent cytoplasm. The background was subtracted for each cell. Only nuclei in sharp focus were measured, and the focus was adjusted until all intact nuclei in the field of view had been measured. Data were collected from eight sections from three different leaves from each treatment.

RESULTS

Effect of Water Stress on Leaf-Blade Elongation

Water stress caused the rate of elongation of the leaf blade to decrease to 51% of the unstressed plants for the 1st d and then to an average of 41% for the next 4 d (Fig. 1). After this, the elongation rate of the unstressed plants slowed down, and that of the stressed plants continued for several more days, with the result that the final blade length of the stressed plants reached 73% of the unstressed plants.

The elongation rate of the second leaf was similarly affected. It was too small to be measured for the first 2 d of the treatment; then between d 3 and 5 it increased from 1.6 to 3.6 mm in length, and stress reduced this by one-half (data not shown).

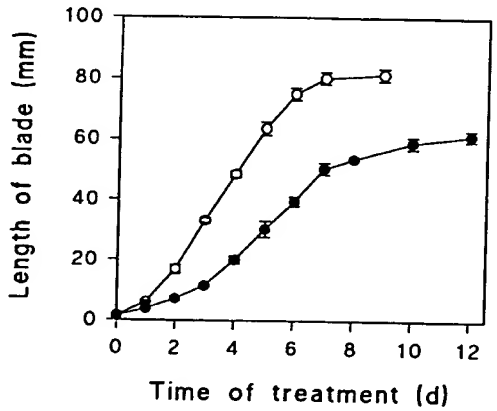

Figure 1. Increase in blade length of the first leaf of wheat in fully hydrated (unstressed; O) and partially hydrated vermiculite at a water potential of −0.3 MPa (stressed;). Treatment commenced at 24 h after imbibition. Bars show the SES of four experiments (n = 30).

Table I. *Developmental pattern of mesophyll cell production in the first leaf of unstressed wheat seedlings*

Measurements started after 24 h of imbibition. The final blade length was reached on d 7. The ligule was present at d 3; therefore, only activity distal to this is recorded. The data are an average of four separate experiments. SES are given for the mitotic index. SES for the blade length are shown in Figure 1. The meristem length is rounded off to the nearest 0.5 mm.

Time	Blade Length	Meristematic Zone Length	Maximum Mitotic Index
d	*mm*		%
0	1.5	1.5	3.6 ± 0.3
1	6	6	3.3 ± 0.3
2	17	8	4.4 ± 0.7
3	33	6	2.5 ± 0.5
4	48	4	1.1 ± 0.5
5	64	2	0.1 ± 0.1
6	75	0	0
7	81	0	0

Developmental Pattern of Mesophyll Cell Production in Unstressed Leaves

The cell-division activity in the unstressed wheat leaf blade throughout its development is summarized in Table I. This shows the length of the meristematic zone of mesophyll cells and the maximum mitotic index based on taking cells clearly in the mitotic phases of metaphase, anaphase, and telophase, but omitting prophase because its recognition was equivocal. After 24 h of imbibition, the mitotic index of mesophyll cells in the first leaf was about 3.5% at the leaf base, declining to 2.5% at the tip. One day later, the mitotic index was again about 3.5% at the base, declining gradually to 0 at the tip of the leaf, which was now 6 mm long. On d 2, mitotic activity in the leaf was at its maximum, both in terms of mitotic index and in the distance from the shoot base over which division occurred (Table I). The ligule started to develop at the base of the shoot meristem on d 2 and by d 3 had moved about 0.5 mm from the base. At this time, when the blade was 40% of its final length, the mitotic activity started to decline (Table I). By d 4, when the blade was about 60% of its final length, mitotic activity had declined further, and by d 5 was observed essentially only below the ligule, i.e. in sheath cells.

Comparison between Stressed and Unstressed Leaves in Spatial Extent and Duration of Mitotic Activity in Mesophyll Cells

After only 1 d of treatment, the mitotic activity in the stressed leaves was much reduced, the maximum mitotic index being only one-half of that in the unstressed controls, and declining to 0 at the tip of the respective leaves from each treatment (Fig. 2A).

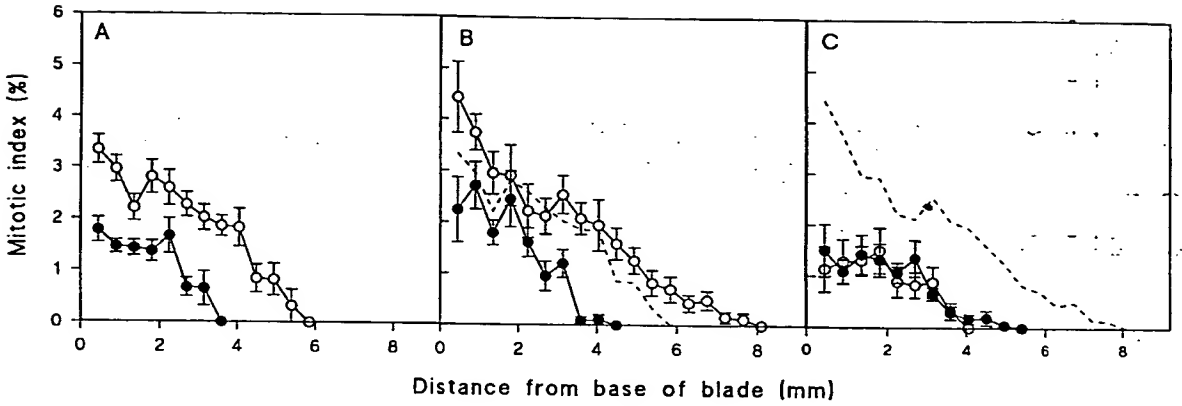

Figure 2. Mitotic index of mesophyll cells as measured in longitudinal sections taken from the blade of the first leaf of wheat seedlings from unstressed (O) and stressed treatments (●) as defined in Figure 1 after 1 d (A), 2 d (B), or 4 d (C) of treatment. Bars show the SES (n = 32). The dotted line on B and C is the "developmental control," i.e. the mitotic index of unstressed leaves of the same size as the stressed leaves (see text for further explanation).

On d 2, when mitotic activity was at its greatest in both unstressed and stressed leaves, the maximum mitotic index in the stressed plants was only one-half of that in the controls, and the zone of division was much shorter (Fig. 2B). Comparison with unstressed leaves at a similar total length (seen in Fig. 2A, and superimposed on Fig. 2B as a dotted line) shows that the meristematic activity in the stressed leaf was still much reduced in comparison with a control leaf at a similar developmental stage.

On d 4 of treatment the division activity in the leaves of unstressed plants was nearing completion, whereas the activity in leaves of the stressed plants was continuing; therefore, the division activity of the two treatments coincided (Fig. 2C). However, the mitotic activity in the stressed leaves was much more reduced than in unstressed leaves of similar length, i.e. d-2 control leaves (superimposed as a dotted line in Fig. 2C). By d 5, the division of blade cells had almost ceased in the unstressed plants and mitotic activity occurred only below the ligule. In the stressed plants mitotic activity continued in blade tissue until d 7, and by d 8 division occurred only below the ligule (data not shown). Therefore, the duration of division was much longer in stressed leaves than in the controls, as was the duration of leaf elongation (Fig. 1).

The total number of cells produced per file was calculated from the final length of the mature leaf blade, which was 81 ± 2 and 59 ± 3 mm for unstressed and stressed plants, respectively (Fig. 1), and from the average length of mature cells, which was 66 ± 3 and 56 ± 2 μm for unstressed and stressed leaves, respectively. This showed that the stressed leaf produced 86% of the cells produced in the unstressed controls along a file. Transverse sections along the blade in both the meristematic zone and in the fully expanded leaves showed that there was no reduction in the number of files of cells in stressed leaves; therefore, the total number of cells in the whole blade of the stressed plants was also 86% of that in the controls.

Effect of Water Stress for 48 h on Cdc2-Like Kinase Activity in the Leaf Base

The basal 6 mm of the shoot was dissected into two segments. The first segment contained the basal 3 mm of the shoot, and the second contained the region from 3 to 6 mm from the base. The kinase was purified from the tissue using $p13^{suc1}$. Affinity chromatography using p13 beads and elution with free $p13^{suc1}$ has previously been used to recover Cdc2-like protein from extracts of leaf meristem cells of wheat (John et al., 1991). Figure 3 shows recovery from different amounts of wheat tissue extract using 20 μL of p13 beads containing 160 μg of $p13^{suc1}$. A linear recovery of activity was obtained for up to 0.06 g fresh weight of tissue from both stressed and unstressed plants. This indicates similar cell contents of the enzyme, since Cdc2 protein forms a semistable complex with $p13^{suc1}$ (Brizuela et al., 1987; Endicott and Nurse, 1995), and the beads become saturated with kinase when all available sites are filled. This causes a plateau in recovery from increasing amounts of extract protein. In the experiments described in Figures 4 to 6, 0.05 g of seedling leaf tissue was used to avoid bead saturation. The data in Figure 3 indicate that the amount of the Cdc2-like protein was not altered by stress but the proportion of active enzyme was reduced.

Figure 4 shows the activity of an affinity-purified Cdc2-like enzyme from 48-h stressed and unstressed wheat seedlings taken up to 12 mm from the base of the leaf. Activity was about 50% lower in extracts from the stressed than the unstressed leaves in both the 0- to 3-mm and 3- to 6-mm segments, and only very low activity was detected 6 to 12 mm from the base.

The Cdc2-like activity in these extracts from the basal shoot tissue (Fig. 4) did not correlate perfectly with mitotic activity shown above for mesophyll cells in stressed and unstressed leaves from plants treated for the same period of time (Fig. 2B). This is because the basal shoot tissue contains a mixture of cell types. Mesophyll cells are the

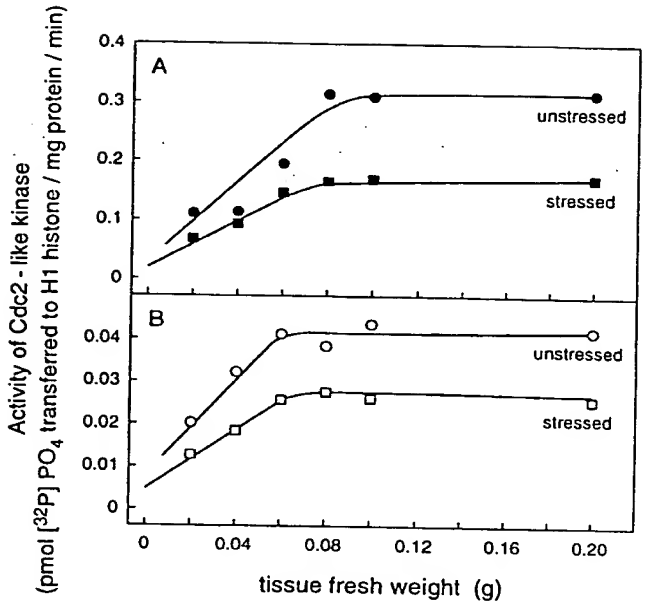

Figure 3. Recovery of Cdc2-like kinase using $p13^{suc1}$ affinity chromatography with 20-μL p13 beads from extracts of leaf segments from unstressed (○, ●) and stressed (□, ■) plants after 48 h of treatment. A, Tissue 0 to 3 mm from leaf base. B, Tissue 3 to 6 mm from leaf base. Activity is related to milligrams of total extracted protein applied to p13 beads for purification prior to assay.

predominant cell type, but there are also cells in the epidermal and vascular tissue. Epidermal cell division in the developing leaf terminates well ahead of mesophyll cell division (because of their larger final length, their numbers are fewer), and division within the vascular tissue proceeds much later and farther from the base than does mesophyll tissue (U. Schuppler, P.-H. He, P.C.L. John, and R. Munns, unpublished observations). Nevertheless, there was strong similarity in the pattern of mitotic activity of mesophyll cells and Cdc2-like enzyme activity in the total shoot base, both in the degree of activity and in the spatial extent.

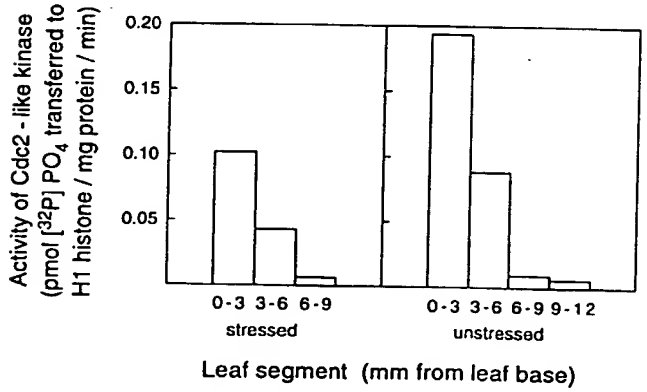

Figure 4. Activity of Cdc2-like kinase purified from extracts of leaf segments taken sequentially from the bases of the leaves of stressed and unstressed plants after 48 h of treatment.

To test whether the lower kinase activity in extracts from the stressed leaves was due to the presence of nonspecific inhibitory or denaturing molecules that could have been elevated by stress, ground, frozen powder from unstressed leaves was mixed with equal amounts from stressed leaves before purification. The kinase activity of this mixture was compared with that of unmixed extracts. Assays were carried out in triplicate. Figure 5 shows that the activity of the mixed extracts from stressed and unstressed plants was the same as the average of the unmixed extracts, indicating that there was no in vitro inhibition of the activity in extracts from stressed tissue.

Speed of Effect of Water Stress on Cdc2-Like Kinase Activity

Kinase activity was measured over time in plants that had been grown under unstressed control conditions in fully hydrated vermiculite for 36 h (rather then 24 h) after imbibition so that the first leaf was big enough to measure at the start of the stress (6–7 mm long). Plants were then transplanted to vermiculite at a water potential of −0.3 MPa or to fully hydrated vermiculite (the transplantation protocol). A response to water stress occurred by 3 h in the basal 3-mm segment, and activity declined to nearly 50% within 6 h (Fig. 6). In the adjacent segment, 3 to 6 mm from the base, reduction in activity was more rapid and detectable from 1 h after stress.

Effect of Water Stress on the Level of Cdc2-Like Protein

To further investigate the conclusion drawn from the experiments with p13-bead saturation (Fig. 3), that amounts of Cdc2-like protein were not altered by stress, the amount of protein was measured directly by probing western blots of total proteins extracted from stressed and control plants. The amount of the Cdc2-like protein was assayed by immunodetection of the PSTAIR region of Cdc2

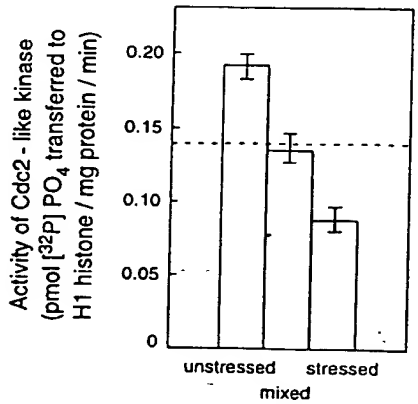

Figure 5. Comparison of activity of Cdc2-like kinase purified from extracts of leaf segments taken from the basal 0 to 3 mm of leaves from unstressed and stressed leaves after 48 h of treatment with that purified from mixed extracts of the same tissues mixed in a 1:1 ratio. The broken line is the calculated mean of stressed and unstressed extracts. Bars show the SES (mean of three separate kinase assays).

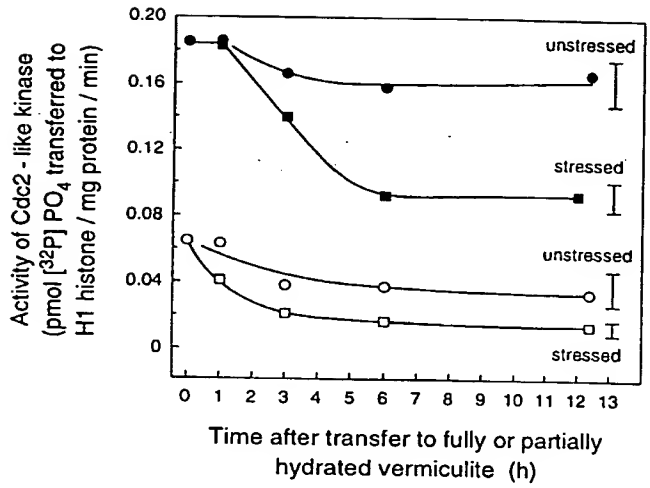

Figure 6. Change with time in Cdc2-like kinase activity in leaves of plants that were first grown on fully hydrated vermiculite for 36 h and then transplanted to pots containing either fully or partially hydrated vermiculite at a water potential of −0.3 MPa (the transplantation protocol). Extracts were made from tissue 0 to 3 mm and 3 to 6 mm from the leaf base from unstressed and stressed plants and were purified before assay. ●, Unstressed, 0 to 3 mm; ○, unstressed, 3 to 6 mm; ■, stressed, 0 to 3 mm; and □, stressed, 3 to 6 mm. Bars show the average SEs for all sampling times (mean of four assays).

proteins. An antibody raised against this peptide revealed a 15-fold higher concentration of Cdc2-like protein relative to total soluble proteins in the meristem region of the wheat leaf compared with other regions of the leaf (John et al., 1990). The same antibody was used to probe western blots of 50-μg protein samples from segments of 48-h stressed and unstressed plants (Fig. 7). Stress had not altered the amount of the Cdc2-like protein in either the first or second segments. This is consistent with the similar saturation of p13 beads by extracts (Fig. 3). The reduction of the kinase activity was therefore not due to a reduction in the level of PSTAIR-containing protein.

Effect of Water Stress on Frequency of G1- and G2-Phase Nuclei of Mesophyll Cells

Water stress may affect Cdc2 kinase activity by slowing the progress between the G1 and S phase or between the G2 and M phase, with consequent changes in the abundance of G1 and G2 phase nuclei. Therefore, the DNA content of nuclei was estimated to establish the frequency of cells in the G1 and G2 phases. Because mitotic activity in mesophyll cells was observed up to 8 mm from the leaf base (Fig. 2), nuclei were measured in the basal 9 mm.

In the unstressed leaf the basal 3 mm, which contains the most actively dividing population of cells, approximately 50% of the cells were in G2 phase (Fig. 8A). In the next 3 mm 38% were in G2 phase (Fig. 8B). At 6 to 9 mm from the base of the leaf only G1 phase cells were observed (Fig. 8C).

In the stressed leaf the basal 3 mm resembled the control in having equal numbers of G1 phase and G2 phase nuclei (Fig. 8D), but in the next 3 mm there was a much reduced frequency of G2 cells, which contributed only 10% of the population (Fig. 8E). The lower incidence of G2 phase cells may reflect accelerated progression to cell differentiation, which in the wheat leaf requires arrest in the G1 phase.

Effect of Water Stress on Phosphorylation State of Cdc2-Like Protein

The early progress of mitosis in eukaryotes depends on the activation of the Cdc2 enzyme, which is determined by a decline in the inactive, Tyr-phosphorylated form. Using an anti-phospho-Tyr antibody, we assayed Tyr-phosphorylated Cdc2-like protein in unstressed and stressed tissue (Fig. 9) sampled after 3 h (lanes 5–9) and then after 48 h (lanes 1–4). As in the earlier time-course experiment, the transplantation protocol was followed (i.e. seedlings were grown in fully hydrated vermiculite for 36 h after imbibition before being transplanted to fully or partially hydrated vermiculite so that they were big enough to measure at the start of the stress). A markedly increased phospho-Tyr level was detected in the protein from the 3- to 6-mm segment after 3 h of stress (Fig. 9), which correlated with the rapid decline in enzyme activity in this segment (Fig. 6). A smaller increase in phospho-Tyr was apparent in the 0- to 3-mm segment (Fig. 9), which correlated with the smaller decline in activity that had developed in this segment by 3 h (Fig. 6). After 48 h of stress, levels of phospho-Tyr in the Cdc2-like protein of both leaf segments from stressed plants were lower than in controls, consistent with cells accumulating in G1 phase (Fig. 8) because of slow cell enlargement or a premature switch to differentiation. While in the G1 phase, Cdc2 protein is not phosphorylated at Tyr (Norbury and Nurse, 1992; Hayles et

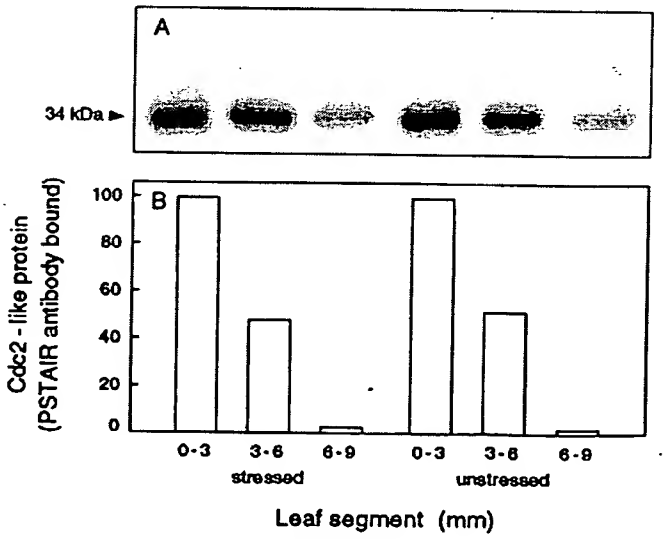

Figure 7. Level of Cdc2-like protein in extracts from basal segments of stressed and unstressed leaf tissue after 48 h of treatment. A, Samples of total soluble protein separated by SDS-PAGE and transferred for western blotting, probed with PSTAIR antibody, and visualized with ^{125}I-labeled anti-rabbit IgG F(ab')$_2$. B, Quantity of PSTAIR-containing 34-kD protein, in relative units measured by phosphor image analysis.

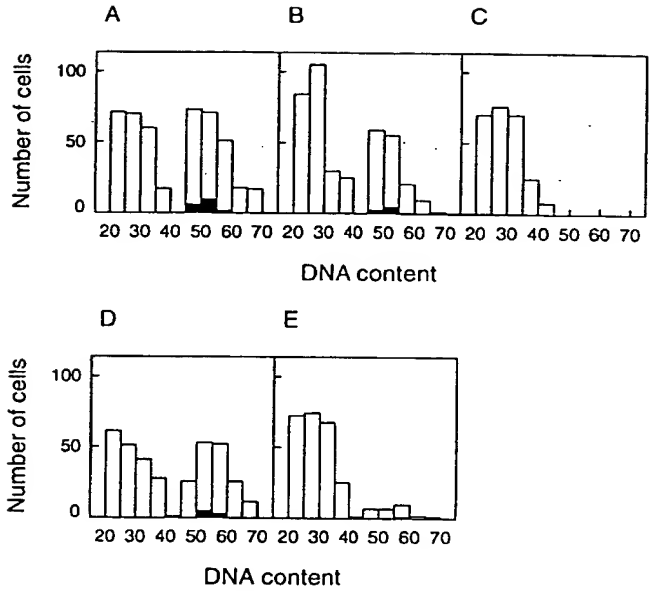

Figure 8. Frequency of mesophyll cell nuclei with DNA contents estimated by Feulgen stain, measured in longitudinal sections of leaves from unstressed and stressed plants after 48 h of treatment. A to C, Unstressed leaves. D to E, Stressed leaves. Mesophyll cells were scored in the basal 0- to 3-mm region of leaves (A and D) and the 3- to 6-mm region (B and E) and the 6- to 9-mm region of unstressed leaves only (C); cells in the 6- to 9-mm region of stressed leaves were entirely nonmitotic and arrested in G1 phase (not shown). Cells with DNA of 20 to 40 relative units were in G1 phase, and those with 45 to 70 units were in G2 phase. Open bars, Total nuclei; solid bars, nuclei in metaphase or anaphase.

al., 1995; Stern and Nurse, 1996), and low activity arises by other mechanisms such as low G1-cyclin levels (Nasmyth, 1966; Sherr, 1996).

DISCUSSION

Effect of Water Stress on Mitotic Activity

Water stress quickly reduced the mitotic activity of mesophyll cells in the meristematic zone and reduced the zone of cell division. A shortening of the zone of cell division in roots by water stress has been reported for sunflower (Robertson et al., 1990b) and maize (Silk, 1992; Sacks et al., 1997). A rapid response of mitotic activity was observed in roots of fava beans exposed to −0.9 MPa PEG (Yee and Rost, 1982), of wheat and pea exposed to about −1.4 MPa mannitol (Bitonti et al., 1991; Bracale et al., 1997), and of sunflowers grown in aeroponic culture with reduced water supply (Robertson et al., 1990a, 1990b). In the studies with fava beans and sunflower, the mitotic index largely recovered after a few days, which suggests that the stress imposed was severe and sudden, and the plants later adjusted to some extent. In our experiments the stress was mild (−0.3 MPa), and the plant response was steady throughout.

Rates of cell division cannot be correctly inferred just from measurements of mitotic activity; there would be no change in the mitotic index or the spatial extent of mitosis if the cell-division rates changed in synchrony with growth rates (Ben-Haj-Salah and Tardieu, 1995; Sacks et al., 1997). However, the decrease in mitotic activity observed here does suggest that cell-division rates might be reduced. From the reduced zone of mitosis and the reduced incidence of mitotic activity in the basal 3 mm of the leaf, we consider it likely that there was a significant reduction in the rate at which cells were being produced. We consider it unlikely that the reduced mitotic index in stressed plants was due to cells passing more quickly through the phase of mitosis than in unstressed plants, in which the expansion phase was less inhibited. As water stress reduces rates of cell expansion in roots (Sharp et al., 1988) and leaves (Spollen and Nelson, 1994; Durand et al., 1995), it is more likely that water stress slows down the rate of cell expansion during the cell-enlargement phases of the cell cycle than that it hastens the mitotic phase in relation to the other phases.

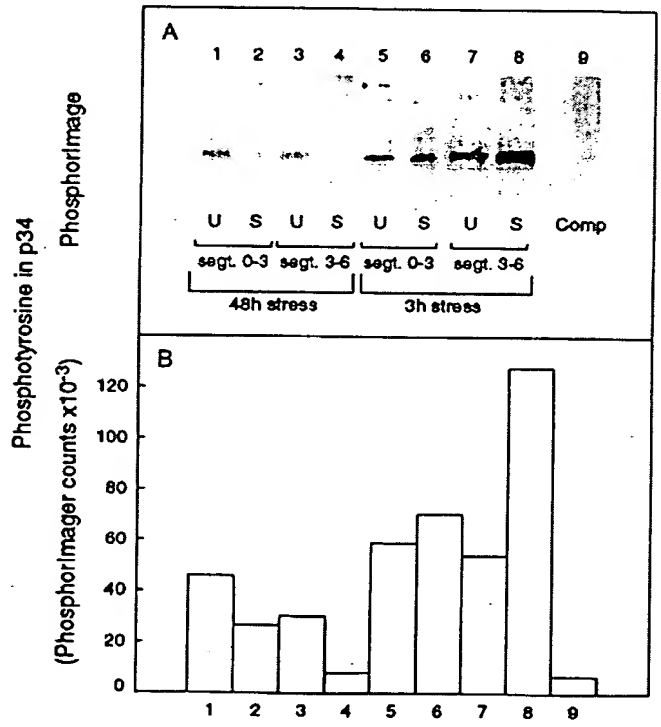

Figure 9. Phospho-Tyr in 34-kD protein that was purified from 300-μg aliquots of total soluble protein by binding and elution with p13 beads. Alternate lanes carry purified enzyme from unstressed (U) and stressed (S) leaves taken from segments 0 to 3 mm (segt. 0–3) or 3 to 6 mm (segt. 3–6) from the base of the leaf. Plants were first grown on fully hydrated vermiculite for 36 h and then transplanted to pots containing either fully or partially hydrated vermiculite at a water potential of −0.3 MPa (the transplantation protocol). Plants were then sampled at 48 h (lanes 1–4) or 3 h (lanes 5–9). Lanes 1 through 8 were probed with anti-phospho-Tyr antibody and in lane 9 this antibody was precompeted (Comp) with 1 mM phospho-Tyr for 1 h before application to a duplicate of lane 8. A, Phosphor image of bound ^{125}I second antibody. B, Quantification of phospho-Tyr in 34-kD protein measured by phosphor image analysis.

A longer period of mitotic activity in the stressed leaves meant that the final number of cells produced was not so different from that in the unstressed leaves (86% of controls), but the time it took to achieve that final number, together with the incremental effect on subsequent leaves, means that growth of the stressed plant would decrease farther behind that of the controls.

Frequency of G1-/G2-Phase Cells

To test for possible preferential effects of a water-stress signal at particular cell-cycle control points, the abundance of cells in the G1 and G2 phases were measured. If progress through a G1 control point was preferentially slowed, we would expect to find an increased number of G1 phase cells and, conversely, preferential mitotic delay would increase the G2 cell population. However, we observed no shift in phase abundance when the first 3 mm of the meristem of stressed plants was compared with the control, although water stress did reduce the kinase activity in this segment and was earlier reported to reduce mitotic activity. The unchanged phase abundance therefore indicates that a mild water stress affected both G1 and G2 control points of the cell cycle in the basal part of the meristem. There is precedent for response at either control point since many plant cells arrest in G1 phase when differentiating, but under stress cells can alternatively arrest in G2 phase and can upon stimulation re-enter the cell cycle from either phase (Van't Hof, 1974, 1985; Bergounioux et al., 1988). The potential for arrest at a G2 control point is supported by the homogenous arrest of tobacco cells in late G2 phase when exposed to auxin without cytokinin (Zhang et al., 1996).

A control acting to prevent progress from G1 phase under stress was detected in cells at the distal margin of the meristem, where G1 phase cells increased from 62 to 90% in the 3- to 6-mm region after 48 h of stress (Fig. 8). Arrest in G1 phase is a component of normal leaf development and occurs as cells cease proliferation and switch to differentiation. Stress caused this to occur in cells nearer the base of the meristem. Both stressed and unstressed leaves showed a gradient of G1-phase cells increasing from 50% at the base to 100% in the cell-differentiation zone, but under stress a greater percentage of G1-phase cells was found in the 3- to 6-mm region. Inhibition of mitotic initiation has been detected in pea roots subjected to severe osmotic stress (Bracale et al., 1997), but the speed with which Cdc2 activity declined was not investigated.

Speed of Response of Cdc2-Like Kinase Activity

To assess the speed with which stress would affect cell-cycle catalysts, we measured an activity denoted as "Cdc2-like." There may have been a contribution from Cdc2 variants, but the measured activity in the $p13^{suc1}$ affinity-purified fraction was predominantly the mitotically active form of Cdc2, since activity of this enzyme is 4 times higher at mitosis than at other times in the cell cycle of plants (John et al., 1993; Zhang et al., 1996) and other organisms (for review, see Nurse, 1990; Stern and Nurse, 1996). We cannot eliminate the possibility of a minor contribution from variants of Cdc2, but activity from such variants has not yet been demonstrated unequivocally in plants. When assayed in animal cells with the H1 histone substrate used here (Tsai et al., 1991), activity from variants was at a low level compared with Cdc2 kinase.

The early decline in the Cdc2-like kinase activity (Fig. 6) indicates that the activation of the enzyme was directly affected by stress and is not consistent with the alternative possibility that slower cell growth might have slowed cell-cycle progress, leading to subsequent reduction in the number of cells approaching mitosis. This possibility must be seriously evaluated because a growth-dependent cell-cycle control point in the G1 phase has been detected in all eukaryotes studied. This control point is termed "start" in yeasts and "restriction point" in animal cells (Jagadish et al., 1977; Pardee, 1989), and an equivalent control point has been detected in unicellular green plants (Donnan and John, 1983; John et al., 1989). Given that the cell population is asynchronous, the decline in mitotic Cdc2 activity developed too rapidly (Fig. 4) to have feasibly resulted from slowed progression from the G1 phase, which would follow a lag equal to the minimum time required to progress from the late G1 control point to mitosis.

The length of the cell cycle of mesophyll cells in cereal leaves has been estimated at about 12 h (MacAdam et al., 1989); therefore, the lag could be approximately 6 h. Only after this time would cells that were in G1 at the time stress was imposed begin to be detected as failing to reach prophase and develop fully active kinase. The observed response (Fig. 6) was very different from this pattern; the response of the kinase activity to mild water stress began early and was completed rapidly. Within 3 h, activity in the 3- to 6-mm segment of stressed plants had declined by nearly 50%. To reinforce this argument, it is possible that the length of the cell cycle in wheat mesophyll cells may be greater than 12 h. The value of 12 h is for tall fescue (MacAdam et al., 1989); mesophyll cell-cycle length has not been assessed in wheat leaves, but epidermal cells of wheat were estimated to have a minimum cell-cycle length of 19 h (Beemster et al., 1997). If mesophyll and epidermal cells of wheat have the same cell-cycle length, then it is even less likely that the decline in Cdc2 activity detected here is merely due to a slowed progression from the G1 phase. We conclude that stress affected the Cdc2 kinase activity.

Activation State of Cd2-Like Kinase

Inactivation of the kinase by stress within 3 h was associated with an increase in the proportion of Tyr-phosphorylated protein, whereas the amount of protein remained constant. This change in phosphorylation state is consistent with the operation of a control during G2 phase that has been intensively studied in yeast and animal cells, in which mitotic activation of Cdc2 depends on the relative activities of inhibitory protein-Tyr kinases, especially Wee1 kinase, and the activating Cdc25 protein Tyr-phosphatase (Russell and Nurse, 1986, 1987; Millar et al., 1991; for review, see Norbury and Nurse, 1992; Lew and Kornbluth, 1996). In plant cells control of Cdc2 kinase activity by Tyr phosphorylation has also been detected at the initiation of

mitosis, when increasing activity correlates with declining content of phospho-Tyr in the protein and with declining capacity for activation of isolated enzyme in vitro with purified Cdc25 enzyme (Zhang et al., 1996). Furthermore, a rate-limiting contribution of Cdc2 enzyme activity in mitosis is indicated by the ability of microinjected plant mitotic kinase to accelerate the prophase events of chromatin condensation: disassembly of the preprophase band and nuclear envelope breakdown (Hush et al., 1996).

The early response to water stress, a decrease in Cdc2-kinase activity within 3 h, must have derived largely from cells in prophase and metaphase, since these contain the most active forms of Cdc2 (Stern and Nurse, 1996; Zhang et al., 1996) and therefore contribute most of the activity in extracts from asynchronous meristems. In this early response the increase in Cdc2 phospho-Tyr and the decrease in Cdc2 activity indicate that the balance of kinase and phosphatase activities that control Cdc2 Tyr phosphorylation is responsive to stress in the plant. The inhibition of Cdc2 was reflected in an increased incidence of G2 phase cells, but as stress continued division was eventually completed, and by 48 h cells had accumulated in G1 phase. There was evidence that low activity of Cdc2 kinase in cells that arrested G1 phase arose from means other than Tyr phosphorylation, since levels of Cdc2 phospho-Tyr were low. This is consistent with evidence in other taxa that during G1 phase, Cdc2 phospho-Tyr is low or absent (Norbury and Nurse, 1992; Stern and Nurse, 1996); therefore, low activity derives from other mechanisms such as limiting G1 cyclin level (Sherr, 1996). However, the rapid inhibition of Cdc2 by Tyr phosphorylation is seen to contribute a fast-acting stress response and indicates that this control, which has been found to be important as a mitotic checkpoint mechanism in all eukaryotes (Nurse, 1994), is also caused by stress in the plant.

Mitotic Progression

A block to prophase is implied by the decline in Cdc2-like kinase activity that we observed under water stress, and there is direct evidence that plant cells do control cell-cycle progress at prophase. Extensive microscopic observation of live plant cells (Hepler, 1985) has revealed that they spend a prolonged but variable time (approximately 2 h) progressing through prophase, as seen by progressively increasing chromatin condensation. However, cells can spontaneously slow or reverse prophase right up to the time of nuclear envelope breakdown (Cleary et al., 1992; Hush et al., 1996). In the microscope-based experimental systems, physiological signals that can cause reversal of progress in prophase have not been investigated, but the present observations that water stress can reduce the activity of Cdc2 enzyme that drives prophase imply that in the intact plant stress can provide such a signal.

Model for Cell-Cycle Control in Water-Stressed Plants

Plants monitor the availability of water (for review, see Davies and Zhang, 1991; Munns and Sharp, 1993), and it is likely that a signaling pathway exists that integrates environmental stress with the control of cell division. Plant hormones have long been postulated as cell-division regulators during developmental and environmental responses (for review, see Evans, 1984; Munns and Sharp, 1993). The availability of water may be indicated by root-derived signals that are transmitted to leaves and affect leaf growth (Passioura, 1988; Davies and Zhang, 1991; Munns and Sharp, 1993). The process by which low soil water potential affects cell division in leaves is not known. It is possible that the water status of the dividing cells is altered, but no measurement of turgor of the dividing cells in leaves has been attempted, and no direct relation between water status and enzyme activity has been discovered. More likely is an altered hormonal balance influenced either by the water status of those cells or by a signal conveyed from roots to shoots.

Exogenous ABA application at high concentrations can reduce mitotic activity in roots (Robertson et al., 1990b; Müller et al., 1994), but this does not prove that endogenous ABA regulates cell-cycle progression. The effects of applied ABA on the proportion of cells in G1 and G2 phase are inconsistent. Applied ABA lengthened the phase of G2 relative to G1 in maize roots (Müller et al., 1994) but not in pea roots (Bracale et al., 1997). The latter study included a water-stress treatment that did lengthen the G2 phase relative to G1, but the stress was a very severe one (0.5 M mannitol). To our knowledge, the effects of exogenous application of ABA on cell-cycle progression in leaves has not been examined. A decreased supply of cytokinins from roots to leaves could conceivably control cell division in leaves of water-stressed plants, although there is no strong evidence to date (for review, see Munns and Cramer, 1996).

Unresolved also is the mechanism by which a root signal impinges on Cdc2 activity. In plant cells auxins are essential in promoting progress through the cell cycle, but cytokinins are most stringently required at the initiation of mitosis (Zhang et al., 1996). The signal could act directly on kinase or phosphatase modifiers of the Cdc2 enzyme but alternatively, could act through a shift in general physiology related to growth rate. Additionally, if plants resemble metazoa in having a specific Cdc2 variant that is dedicated to S-phase events and is under the control of inhibitory Tyr phosphorylation, this phosphorylation could also underlie the accompanying decline in G1-/S-phase progression. We are currently unable to test this because kinase recovered by $p13^{suc1}$ affinity is predominantly Cdc2 protein.

In fission yeast the growth rate clearly influences the timing of mitosis and, although the metabolites involved have not been identified, the mechanism is based on altered timing of Tyr phosphorylation in Cdc2 kinase (Russell and Nurse, 1986, 1987; Moreno et al., 1990; Lew and Kornbluth, 1996; Sveiczer et al., 1996). The requirement for cytokinin in plant cell division can be entirely met by expression of the Cdc2 Tyr-phosphatase Cdc25 (K. Zhang, L. Deiderich, F.J. Sek, P.J. Larkin, and P.C.L. John, unpublished observations), and the same Cdc2 phosphorylation is seen to occur under stress in the current study. However, it has not yet been tested whether this effect occurs solely through a mechanism that can be reversed by Cdc25 activ-

ity. A similar phosphorylation mechanism in wheat could account for the decline in mitosis.

Our model for the effect of mild water stress on leaf growth is that stress affects the cell cycle at control points in G1 phase and at late G2 phase. We propose that water stress induces a signal that increases the phosphorylation of Tyr at the active site of Cdc2 kinase and that this results in a predominance of the inactive form of the enzyme, with consequent inhibition of progression into mitosis, which is dependent on high activity of the enzyme. Since there is no long-term accumulation of cells in G2 phase, we deduce that there is also a slowing of progress from the G1 phase to the G2 phase, and in cells at the distal margin of the meristem this accumulation of cells in G1 phase accentuates a switch to cell differentiation that reduces meristem size.

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CDK-related protein kinases in plants

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Abstract

Cyclin-dependent kinases (CDK) form a conserved superfamily of eukaryotic serine-threonine protein kinases, which require binding to a cyclin protein for activity. CDK are involved in different aspects of cell biology and notably in cell cycle regulation. The comparison of nearly 50 plant CDK-related cDNAs with a selected set of their animal and yeast counterparts reveals five classes of these genes in plants. These are described here with respect to their phylogenetic, structural and functional properties. A plant-wide nomenclature of CDK-related genes is proposed, using a system similar to that of the plant cyclin genes. The most numerous class, *CDKA*, includes genes coding for CDK with the PSTAIRE canonical motif. *CDKB* makes up a class of plant-specific CDK divided into two groups: *CDKB1* and *CDKB2*. *CDKC*, *CDKD* and *CDKE* form less numerous classes. The *CDKD* class includes the plant orthologues of metazoan CDK7, which correspond to the CDK-activating kinase (CAK). At present, no functional information is available in plants for *CDKC* and *CDKE*.

Introduction

Cyclin-dependent protein kinases (CDK) are now widely recognized as key players at various checkpoint controls of the eukaryotic cell cycle (Nigg, 1995; Morgan, 1997; Mendenhall and Hodge, 1998). Their importance was realized nearly ten years ago from independent genetic approaches in yeast and biochemical studies of mitotic controls in fertilized eggs of marine invertebrates. These studies revealed that the same serine-threonine protein kinase is involved both in G_1-S and in G_2-M transitions in yeast (the *cdc28* gene of *Saccharomyces cerevisiae* and its orthologue *cdc2* of *Schizosaccharomyces pombe*), and is the main component of the mitosis-promoting factor in fertilized eggs (Norbury and Nurse, 1992). The functional definition of a CDK is its requirement of cyclin binding for activity. In eukaryotes, CDK and cyclins form large superfamilies, so that a very large number of CDK-cyclin complexes can occur with various substrate specificity at various locations within the cell and at various time points within the cell cycle. The CDK-cyclin complexes ensure a number of functions of which the direct regulation of the cell cycle is the most thoroughly characterized. The substrates of CDK-cyclin complexes include transcriptional regulators, cytoskeleton, nuclear matrix, nuclear membrane proteins as well as other cell cycle proteins. The functions of CDK in the cell cycle have been extensively reviewed (Nigg, 1995; Morgan, 1997; Mendenhall and Hodge, 1998) and will not be considered in detail in this review. Other functions include the response to

nutrient starvation (Lenburg and Oshea, 1996; Andrews and Measday, 1998) and the control of transcription (Dynlacht, 1997; Andrews and Measday, 1998).

The discovery of a conserved set of genes controlling the eukaryotic cell cycle contributed largely to the molecular unravelling of the plant cell cycle (Francis and Halford, 1995; Jacobs, 1995; Sopory and Munshi, 1998; Mironov *et al.*, 1999). Genes for CDK and mitotic cyclins were the first cell cycle genes characterized in plants (Colasanti *et al.*, 1991; Ferreira *et al.*, 1991; Hata *et al.*, 1991; Hirt *et al.*, 1991; Hemerly *et al.*, 1992), and many other conserved cell cycle regulators have now been described (Soni *et al.*, 1995; Grafi *et al.*, 1996; Xie *et al.*, 1996; Deveylder *et al.*, 1997; Wang *et al.*, 1997; Porat *et al.*, 1998; Sun *et al.*, 1999). However, although eukaryotic CDK and cyclins are highly conserved, a simple extrapolation from animal and yeast proteins is not sufficient to fully assess their functionality in plants. The plant cell cycle displays a number of unique characteristics including cytoskeleton organization and cytokinesis (Fowler and Quatrano, 1997; Heese *et al.*, 1998), reversibility of terminal differentiation and control by plant hormones (Jacobs, 1995; Shaul *et al.*, 1996; Coenen and Lomax, 1997; Meyerowitz, 1997). The general occurrence of large superfamilies of cyclins and CDK in eukaryotes is a further complication for a single, unified model of cell cycle controls, especially because these gene families have not followed the same evolution in all kingdoms. In the past few years, a rapid increase in the number of identified plant cyclin sequences has enabled the establishment of a structural classification and the proposal of a standardized nomenclature consisting of two groups of B-type cyclins (*CYCB1* and *CYCB2* genes) and three groups of A-type (*CYCA1*, *CYCA2* and *CYCA3)* and D-type cyclins (*CYCD1*, *CYCD2* and *CYCD3* genes) (Renaudin *et al.*, 1996). This analysis has revealed a number of structural peculiarities of plant cyclins and the proposed classification tends to fit major functional differences, even though some groups may still be functionally heterogeneous.

Although their number has increased at a lower rate than cyclins, plant CDK now represent nearly 50 different sequences in more than 20 species. As previously observed for cyclins, plant CDK display both a high degree of homology and some structural and functional differences compared with animal and yeast CDK (Jacobs, 1995; Mironov *et al.*, 1999; Umeda *et al.*, 1999). The absence of a common nomenclature is a further obstacle to a general understanding of CDK functions in plants (Meyerowitz, 1997; Dudits *et al.*, 1998). The purpose of this review is to compare plant CDK described to date and their relationships with established classes of animal and yeast CDK. A plant-wide nomenclature of CDK, similar to the one previously established for plant cyclins, is proposed.

Structure-function relationship of CDK

Eukaryotic serine-threonine and tyrosine protein kinases encompass a 300 amino acid catalytic core which shares considerable primary, secondary and tertiary structure similarity (Hanks *et al.*, 1988; Goldsmith and Cobb, 1994). The CDK belong to the CMGC group, which also includes MAPK, GSK-3 and CKII families. Central to their functional properties is the positive regulation of CDK by cyclin binding and by phosphorylation (on Thr-160 in human CDK2), and their negative regulation by cyclin-dependent kinase inhibitors (CKI) and by phosphorylation (on Thr-13 and Tyr-15 in human CDK2). CDK are basically composed of a catalytic core with short amino- and carboxy-terminal extensions. The determination of the crystal structure of human CDK2 (DeBondt *et al.*, 1993; Jeffrey *et al.*, 1995; Russo *et al.*, 1996a, b) and CDK6 (Brotherton *et al.*, 1998; Russo *et al.*, 1998) has shown that the overall structure of CDK has an inherent structural flexibility that is central to their regulation. The structural features of human CDK2 are presented in Figure 1. The catalytic core of CDK is organized as a small N-terminal lobe of ca. 85 residues with mostly β-sheet structure, and a large C-terminal lobe containing six α-helices (DeBondt *et al.*, 1993). Between the two lobes, a 40 amino acid portion constitutes a deep catalytic cleft with ATP phosphate and substrate binding sites and a T-loop which has significant freedom with respect to binding and phosphorylation. When the kinase is unbounded and unphosphorylated, the highly conserved residues involved in ATP phosphate orientation and magnesium coordination (the backbone amides of the glycine-rich loop between β1 and β2, Lys-33, Glu-51, Asn-132, Asp-145) (Figure 1) are not properly positioned, and several residues of the T-loop prevent effective binding of the substrate protein and access to the γ-phosphate of ATP.

CDK activation by cyclin

Binding to cyclin is a process required for CDK activity. Crystallographic analysis of the CDK2-cyclin

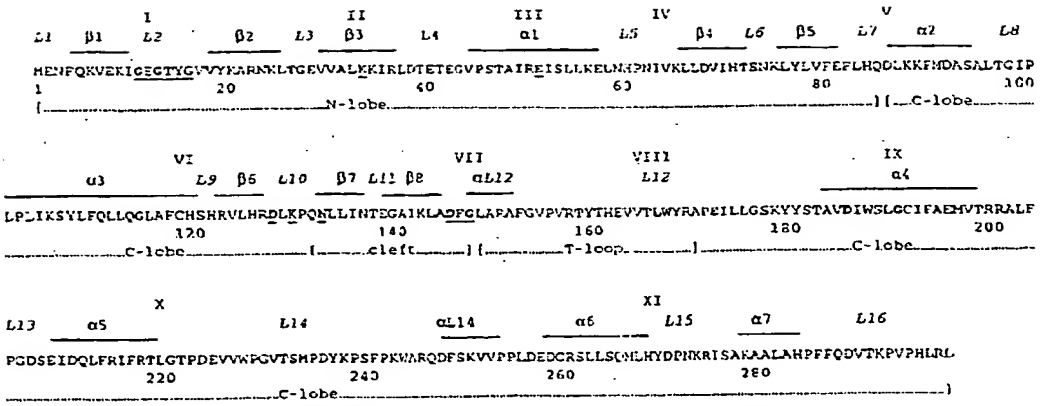

Figure 1. Structural characteristics of human CDK2. Secondary structural elements, α-helices, β-strands and linker regions are numbered according to DeBondt *et al.* (1993). The 11 domains conserved in all serine-threonine and tyrosine protein kinases are numbered I–XI above the sequence and the fifteen most conserved residues in these kinases are in bold characters (Hanks *et al.*, 1988). The positions of the N-terminal lobe, the C-terminal lobe, the catalytic cleft and the T-loop are indicated, and highly conserved residues involved in the ATP binding site are underlined (DeBondt *et al.*, 1993).

A complex (Jeffrey *et al.*, 1995) has shown that the first repeat of the cyclin fold (i.e. the cyclin box which is the most conserved part of the cyclin core) is the key element at the cyclin-CDK2 interface. It binds the CDK2 protein within the two lobes and the T-loop. The most extensive binding occurs within the α1 helix of CDK2, which contains a highly conserved PSTAIRE motif, the CDK hallmark. The binding induces a rotation of the helix into the catalytic cleft, which properly realigns active site residues such as Glu-51. Extensive interactions also occur between cyclin and T-loop residues inducing melting of the αL12 helix and relieving the block it exerts on the catalytic site, allowing the α1 helix of CDK2 to move deeper into the catalytic cleft.

CDK regulation by phosphorylation and by CKI

The activation of CDK2 and related CDK, but not CDK5 or CDK7, requires the phosphorylation of Thr-160 located at the entrance of the catalytic cleft. The phosphorylation of Thr-160 induces conformational changes of the T-loop and of the C-terminal lobe of CDK2 (Russo *et al.*, 1996b). This stabilizes the T-loop in an active position by ionic interactions with several cationic residues (Arg-50, Arg-126, Arg-150), in accordance with the situation found for cAMP-dependent protein kinase where a similar autophosphorylated residue is present within a cationic pocket. The conformational changes induced by phosphate binding affect both the cyclin interface and the putative substrate-binding site. The phosphorylation of Thr-14 and Tyr-15, which is inhibitory to CDK2 activity, brings the side chain of Tyr-15 deeper into the cleft within hydrogen-bonding distance of Glu-51. In this way, ATP fixation is inhibited and protein substrate binding is blocked. CDK activity may be negatively regulated by two families of inhibitors. The KIP/CIP family has a broad specificity towards CDK-cyclin complexes. It can bind both monomers but the binding to the complex occurs with higher affinity. The KIP/CIP inhibitors contain a 65 amino acid portion, sufficient for their activity, which interacts with a large area on the surface of cyclin and CDK, as shown from a crystallographic study of the $p27^{KIP1}$-cyclin A-CDK2 complex (Russo *et al.*, 1996a). In CDK2, the region of binding is the N-terminal lobe in which extensive structural changes alter the ATP-binding glycine loop and cause large conformational changes in and around the catalytic cleft. In animals, the INK4 family of CDK inhibitors is more specific for the CDK4 and CDK6 kinases involved along with cyclin D in the G_1-S transition. Their binding to the two lobes of the kinases prevents both cyclin and ATP binding (Brotherton *et al.*, 1998; Russo *et al.*, 1998).

Diversity and nomenclature of yeast and animal CDK

The completion of budding yeast genome sequencing has revealed the presence of 5 CDK among the 113 conventional protein kinases of this unicellular eukaryote (Hunter and Plowman, 1997; Andrews and Measday, 1998). CDC28 is the only CDK to play an essential role in cell cycle regulation (Mendenhall and Hodge, 1998). PHO85 and KIN28 are involved not only in cell cycle control but also in other aspects of cellular regulation (Lenburg and Oshea, 1996; Andrews and Measday, 1998). These three kinases possess the canonical AIRE motif in α1 helix. An additional member, SSN3/SRB10, has no apparent role in the cell cycle but forms a complex with cyclin SRB11 as part of the RNA polymerase II holoenzyme. The fifth CDK, CTK1, is a relatively divergent kinase identified as part of the RNA polymerase II carboxy-terminal domain kinase. CTK1 has a SIRE sequence in α1 helix and it binds CTK2, a cyclin-related protein. On the contrary, the CDK-activating kinase CAK1 is a kinase that is structurally more related to CDK1-4 but has been shown to be active as a monomer in the absence of cyclin binding (Espinoza *et al.*, 1996; Kaldis, 1999). Accordingly, it displays an AKFE sequence in the α1 helix and is not to be classified as a true CDK, but only as a CDK-related protein.

In man, the much higher number of CDK compared to yeast is a direct consequence of the more complex cell cycle controls in metazoans. Human CDK have been named in the order of their discovery (e.g. CDK1, CDK2, etc.). The nomenclature in other animal species follows the human system. The current number of human CDKs is 12 (Espinoza *et al.*, 1996; Morgan, 1997; Defalco and Giordano, 1998; Kaldis, 1999). Many of these human CDKs are alternatively described according to their sequence in α1 helix (Meyerson *et al.*, 1992). In this α1 helix, the canonical PSTAIRE sequence is only observed for CDK1 to CDK3. As a consequence, altered sequences in this domain may reflect the binding to particular cyclins or beyond a certain degree of alteration, the absence of binding to a cyclin-like protein. Thus there is still uncertainty as to which extent all human kinases with altered sequences in helix α1 are true CDK or only CDK-related kinases. The same uncertainty will be encountered with plant CDK which we shall consider to be CDK-related kinases when there is no information on cyclin binding.

Plant CDK are organized in five major gene families

A total of 46 putative plant CDK have been described so far in 23 species of algae, gymnosperms and angiosperms. The complete sequences of these proteins and of a defined set of yeast and animal CDK were compared (Table 1, Figure 2). Plant CDKs are encountered in five evolutionarily conserved classes, which indicate a high level of complexity of the evolution of this superfamily of protein kinases (Table 1, Figure 2). In accordance with previous attempts to classify plant *CDK* genes (Mironov *et al.*, 1999), we have assigned the names *CDKA* to *CDKE* to the five classes of plant *CDK* genes, and we analyse below the structural characteristics of each class. We show that this structurally grounded classification is consistent with the functional characteristics of plant CDK available in the literature.

Plant CDKA

CDK with the canonical PSTAIRE motif form the most numerous class, with representatives in the three kingdoms. Although it displays a slightly altered PSTALRE motif, the *Dictyostelium discoidum* CDC2 homologue is able to complement yeast mutants (Michaelis and Weeks, 1992), and is thus included in this class (Figure 2). The 31 plant CDK present in this class are more closely related to yeast (Sc;CDC28, Sp;CDC2) and human (CDK1, -2, -3) CDK than to plant CDK of the same species but from other groups. It is proposed that the PSTAIRE plant CDK be considered to form one gene family named *CDKA*, in accordance with Burssens *et al.* (1998) and Mironov *et al.* (1999). The complementation of temperature-sensitive mutants in yeast *cdc2/cdc28* genes, which was the first evidence for plant CDK functionality, was successful only with plant CDK from the CDKA group (Colasanti *et al.*, 1991; Ferreira *et al.*, 1991; Hirt *et al.*, 1993). In *S. cerevisiae*, the CDC28 protein alone is involved in G_1/S and G_2/M controls (Mendenhall and Hodge, 1998) whereas in animals, CDK1, -2, -3, all belonging to the same group as plant CDKA, ensure distinct functions from G_1 to mitosis (Morgan, 1997). At the transcript and protein levels, plant *CDKA* do not show preferential expression at any phase of the cell cycle and are detected at low levels in non-dividing tissues (Martinez *et al.*, 1992; Hemerly *et al.*, 1993) (Figure 3). Altogether, these results led to the proposal of a dual

Table 1. A list of plant CDK-related kinases. The sequences have been renamed according to the new nomenclature. Accession numbers in EMBL/GenBank/DDBJ/PIR databases are indicated.

New gene name	Old gene name	Species	Accession	Reference
CDKA class				
Allce;*CDKA;1*	cdc2	*Allium cepa*	DDBJ AB005033	Okushima *et al.*, unpublished
Antma;*CDKA;1*	Amcdc2a	*Antirrhinum majus*	EMBL X97637	Fobert *et al.*, 1996
Antma;*CDKA;2*	Amcdc2b	*Antirrhinum majus*	EMBL X97638	Fobert *et al.*, 1996
* Arath;*CDKA;1*	Atcdc2a	*Arabidopsis thaliana*	GenBank M59198	Ferreira *et al.*, 1991
Betvu;*CDKA;1*	cdc2	*Beta vulgaris*	GenBank Z71702	Kirby *et al.*, unpublished
Brana;*CDKA;1*	cdc2	*Brassica napus*	GenBank U13365	Szarka and Moloney, unpublished
Chera;*CDKA;1*	cdc2	*Chenopodium rubrum*	EMBL Y10160	Renz *et al.*, unpublished
Glyma;*CDKA;1*	cdc2-S6	*Glycine max*	GenBank M93140	Miao *et al.*, 1991
Glyma;*CDKA;2*	cdc2-S5	*Glycine max*	GenBank M93139	Miao *et al.*, 1991
Lyces;*CDKA;1*	Lecdc2A-1	*Lycopersicon esculentum*	EMBL Y17225	Joubès *et al.*, 1999
Lyces;*CDKA;2*	Lecdc2A-2	*Lycopersicon esculentum*	EMBL Y17226	Joubès *et al.*, 1999
Medsa;*CDKA;1*	cdc2MsA	*Medicago sativa*	GenBank M58365	Magyar *et al.*, 1997
Medsa;*CDKA;2*	cdc2MsB	*Medicago sativa*	EMBL X70707	Hirt *et al.*, 1993
Nicta;*CDKA;1*	Ntcdc2-1	*Nicotiana tabacum*	GenBank L77082	Qin *et al.*, unpublished
Nicta;*CDKA;1*	Ntcdc2-2	*Nicotiana tabacum*	GenBank L77083	Qin *et al.*, unpublished
Nicta;*CDKA;3*	cdc2Nt1	*Nicotiana tabacum*	DDBJ D50738	Setiady *et al.*, 1996
Orysa;*CDKA;1*	cdc2Os-1	*Oryza sativa*	EMBL X60374	Hashimoto *et al.*, 1992
Orysa;*CDKA;2*	cdc2Os-2	*Oryza sativa*	EMBL X60375	Hashimoto *et al.*, 1992
Petcr;*CDKA;1*	cdc2	*Petroselinum crispum*	GenBank L34206	Logemann *et al.*, 1995
Pethy;*CDKA;1*	cdc2	*Petunia hybrida*	EMBL Y13646	Trehin *et al.*, 1998
Picab;*CDKA;1*	cdc2Pa	*Picea abies*	EMBL X77680	Kvarnheden *et al.*, 1995
Pinco;*CDKA;1*	cdc2Pnc	*Pinus contorta*	EMBL X80845	Kvarnheden *et al.*, 1995
Pissa;*CDKA;2*	cdc2	*Pisum sativum*	DDBJ AB000318	Shimizu and Mori, 1998
Solru;*CDKA;2*	cdc2	*Solanum tuberosum*	GenBank U53510	Campbell and Sutile, unpublished
Sesra;*CDKA;1*	Srcdc21	*Seshania rostrata*	EMBL Z75651	Geoumachtig *et al.*, unpublished
Triae;*CDKA;1*	cdc2TaA	*Triticum aestivum*	GenBank U23409	Dong and John, unpublished
Triae;*CDKA;2*	cdc2TaB	*Triticum aestivum*	GenBank U23410	Dong and John, unpublished
Vigae;*CDKA;1*	cdc2	*Vigna aconitifolia*	GenBank M99497	Hong *et al.*, 1993
Vigun;*CDKA;1*	cdc2	*Vigna unguiculata*	EMBL X89400	Kmuse, unpublished
Zeama;*CDKA;1*	cdc2A	*Zea mays*	PIR A40444	Colasanti *et al.*, 1991
Zeama;*CDKA;2*	cdc2B	*Zea mays*	PIR B40444	Colasanti *et al.*, 1991
CDKB class				
Antma;*CDKB1;1*	Amcdc2c	*Antirrhinum majus*	EMBL X97639	Fobert *et al.*, 1996
Antma;*CDKB2;1*	Amcdc2d	*Antirrhinum majus*	EMBL X97640	Fobert *et al.*, 1996
Arath;*CDKB1;1*	Atcdc2b	*Arabidopsis thaliana*	DDBJ D10851	Imajuku *et al.*, 1992
Dunte;*CDKB;1*	cdc2	*Dunaliella tertiolecta*	GenBank AAD08721	Lin and Carpenter, unpublished
Medsa;*CDKB1;1*	cdc2MsD	*Medicago sativa*	EMBL X97315	Magyar *et al.*, 1997
Medsa;*CDKB2;1*	cdc2MsF	*Medicago sativa*	EMBL X97317	Magyar *et al.*, 1997
Mescr;*CDKB2;1*	cdc2	*Mesembryanthemum crystallinum*	DDBJ AB015182	Fukuhara and Bohnert, unpublished
Orysa;*CDKB;1*	cdc2Os-3	*Oryza sativa*	DDBJ D64036	Kidou *et al.*, 1992
CDKC class				
Arath;*CDKC;1*	cdc2cAt	*Arabidopsis thaliana*	GenBank T42526	Burssens *et al.*, 1998; Newman *et al.*, 1994
Arath;*CDKC;2*	cdc2dAt	*Arabidopsis thaliana*	GenBank T20748	Burssens *et al.*, 1998; Newman *et al.*, 1994
Medsa;*CDKC;1*	cdc2MsC	*Medicago sativa*	EMBL X97314	Magyar *et al.*, 1997
Pissa;*CDKC;1*	Ps2cdc2	*Pisum sativum*	EMBL X56554	Feiler and Jacobs, 1991

Table 1. Continued

New gene name	Old gene name	Species	Accession	Reference
CDKD class				
Arath;*CDKD;1*	CAK2At	*Arabidopsis thaliana*	–	Umeda *et al.*, unpublished
Orysa;*CDKD;1*	R2	*Oryza sativa*	EMBL X58194	Hata, 1991
CDKE class				
Medsa;*CDKE;1*	cdc2MsE	*Medicago sativa*	EMBL X97316	Magyar *et al.*, 1997

function for plant CDKA during both S and M phase progression (Colasanti *et al.*, 1991; Ferreira *et al.*, 1991; Hirt *et al.*, 1993) and for their involvement in cell proliferation and the maintenance of cell division competence in differentiated tissues during plant development (Martinez *et al.*, 1992; Hemerly *et al.*, 1993).

Figure 4 presents an enlarged phylogenetic tree of 31 plant *CDKA* from 22 different species of gymnosperms and angiosperms. The most noticeable feature of Figure 4 is that the clusters of sequences are strictly related to taxonomy. The two CDKA from the Pinaceae family are clustered together on a separate branch of the phylogenetic tree. A truly distinct branch for gymnosperms was convincingly obtained when the CDKA pseudogene sequences from *Picea abies* (Kvarnheden *et al.*, 1995, 1998) were included (data not shown). Within angiosperms, the eight clusters of sequences represent two families of monocotyledons (Gramineae and Liliaceae), and six families of dicotyledons (Brassicaceae, Chenopodiaceae, Leguminoseae, Scrophylariaceae, Solanaceae and Umbelliferae). There is thus no evidence for the occurrence of groups of *CDKA* such as suggested for *CDKB* (see below). The fact that in eight genera, two different *CDKA* genes are present in a given species indicates gene duplication events which have occurred after the divergence of families. However these events may have been anterior to the divergence of genera, as the two sequences from a same species, e.g. *Oryza* and *Triticum*, *Medicago* and *Glycine* spp., are less closely related to each other than to sequences of another genus in the same family (Figure 4). Conversely, the two closely related maize *CDKA* genes are indicative of a recent duplication event (Figure 4). The occurrence of two *CDKA* genes in several species may indicate functionally distinct proteins. This is suggested by the finding that different *S. cerevisiae cdc28* mutant alleles blocked respectively at G_1/S and G_2/M transitions were complemented by two distinct *CDKA* genes in alfalfa (Medsa;*CDKA1;1* and Medsa;*CDKA2;1*) (Hirt *et al.*, 1993). We propose identifying the member number of a *CDKA* gene in any species by Arabic numerals allocated sequentially. The same rule could hold for any newly characterized *CDKB–CDKE* gene. It must be clear, however, that *CDKA*;1 from two species are not systematically orthologous.

The three *Nicotiana tabacum CDKA* genes are clustered together on the same branch. In addition to the amphidiploid character of tobacco, it is interesting to note that these three sequences come from two different strains, namely Xanthi and Samsun NN (Table 1, Figure 4). The two *CDKA* genes from *Antirrinum majus* (Scrophylariaceae) are not found in the same cluster: one is closely related to Solanaceae sequences whereas the other (Amcdc2b) appears distantly related to other *CDKA* sequences. Characterization of more *CDKA* from Scrophylariaceae is needed to explain this unusual situation.

The 31 plant CDKA have a highly conserved amino acid sequence, with 89% similarity (Figure 5). The fifteen most conserved residues of eukaryotic serine-threonine and tyrosine protein kinases are conserved in plant CDKA, including the residues involved in the ATP-binding site and in regulatory phosphorylation (Figure 5). The residues involved in defining the consensus phosphorylation site of CDK, i.e. SPXK, where S is the phosphoacceptor, are also conserved in plant CDKA (Figure 5). Over a total of 38 residues conserved in animal and yeast CDK, and which are exposed to solvent in free human CDK2, i.e. susceptible to bind regulatory proteins (DeBondt *et al.*, 1993), plant CDKA have only three changes compared with human CDK2 (data not shown). These data suggest a high degree of functional similarity within the PSTAIRE group of CDK, which is reinforced by the finding that the theoretical structure of

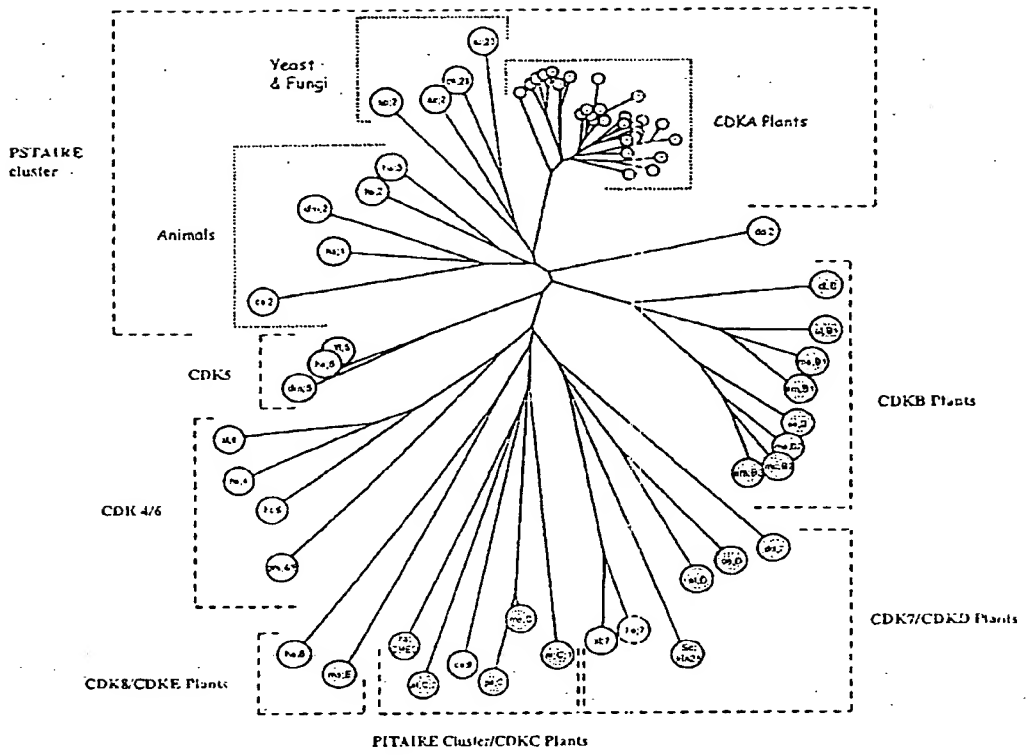

Figure 2. A phylogenetic tree of eukaryotic cyclin-dependent protein kinases. The sequences of the plant CDK-related kinases listed in Table 1, together with a selected set of animal and yeast CDK, were aligned by using the multiple alignment program CLUSTAL W version 1.7. The Phylip package was used for the construction of phylogenetic trees and for the comparison of parsimony and distance (Neighbour Joining algorithm) methods. The robustness of the trees was assessed by the bootstrap method. The length of lines is proportional to the genetic distances between nodes. For clarity, species are indicated by the first two letters of the generic and specific names. Classes of CDK are identified as described in the text. Plant CDKs are indicated in grey circles. *Caenorhabditis elegans*: ce;2, CDK2, P34556; ce;9, CDK9, P46551. *Drosophila melanogaster*: dm;2, CDK2, P23572; dm;4/6, CDK4/6, X99510; dm;5, CDK5, P48609. *Homo sapiens*: hs;1, CDK1, P06493; hs;2, CDK2, P24941; hs;3, CDK3, Q00526; hs;4, CDK4, P11802; hs;5, CDK5, Q00535; hs;6, CDK6, Q00534; hs;7, CDK7, P50613; hs;8, CDK8, P49336; hs;CHED, CHED kinase, M80529. *Xenopus laevis*: xl;5, CDK5, P51166; xl;6, CDK6, S57926; xl;7, CDK7, P20911. The two *Dictyostelium discoideum* CDKs are: dd;2, CDK2, P34112; dd;7, CDK7, P54685. The yeast and fungal CDKs are: *Ajellomyces capsulatus* ac;2, cdc2, P54119; *Candida albicans* ca;23, cdc23; *Saccharomyces cerevisiae*: sc;28, cdc28, P06546; sc;kin28, kin28, P06242; *Schizosaccharomyces pombe* sp;2, cdc2, P04551.

the alfalfa CDKA;1 kinase is similar to that of human CDK2 (Dudits *et al.*, 1998). However, limited data are available about the binding of plant CDKA kinases to cyclins. Two recent reports have shown using different methods that CDKA kinases can bind plant cyclin D: a two-hybrid screen showed that the *Arabidopsis* CDKA;1 protein binds At;CycD1;1 cyclin (Deveylder *et al.*, 1997), and immunoprecipitation with anticyclin antibody showed that the tobacco CDKA;3 binds Nt;CycD3;1 (Nakagami *et al.*, 1999). The CDKA/CycD complex was able to phosphorylate *in vitro* the tobacco homologue of Rb, in a way compatible with a role at the G_1-S transition (Nakagami *et al.*, 1999). The recent discovery of a high level of expression of two tobacco CycD cyclins during mitosis could also suggest that, even at that time, CDKA

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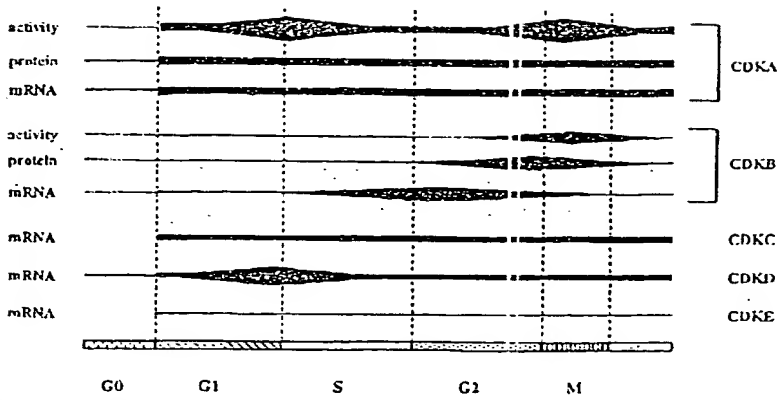

Figure 3 Expression of plant CDK during the different phases of the cell cycle. The level of transcript, protein and activity is qualitatively represented by the thickness of the areas. Dots indicate an absence of detected mRNA, protein or measured activity (adapted from Mironov *et al.*, 1999).

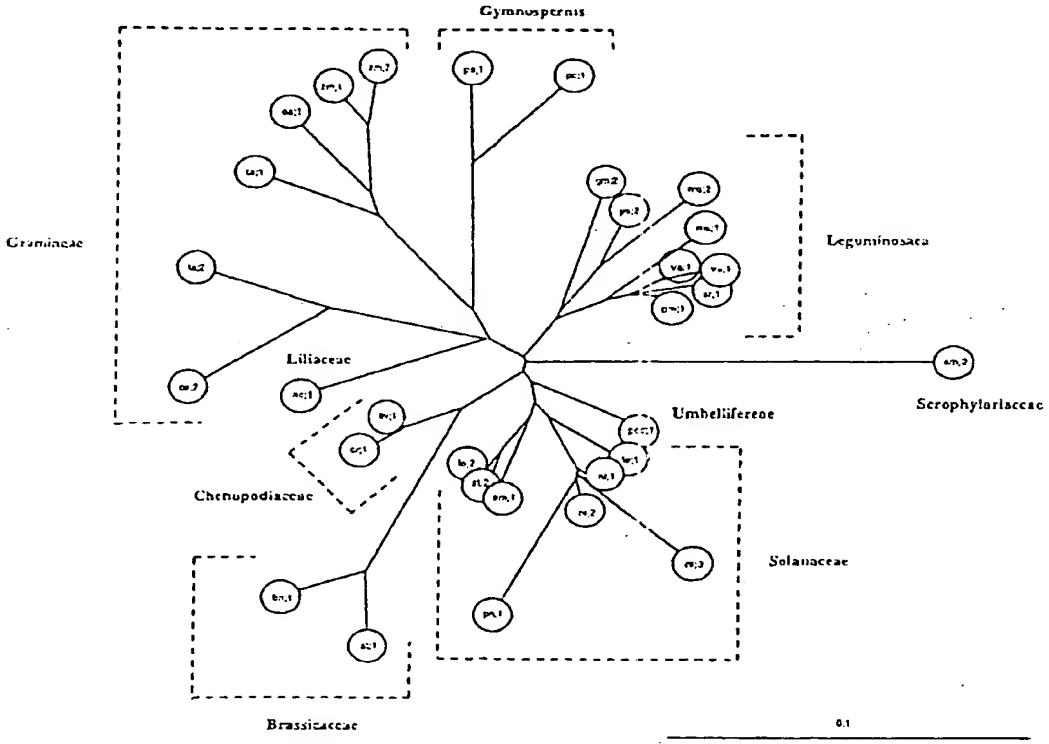

Figure 4. A phylogenetic tree of plant CDKA protein kinases. The tree includes the sequences described in Table 1. (See Figure 2 for details.) Species are indicated by the first letter of the genus and species names. Arabic numerals indicate member numbers. Clusters of sequences including gymnosperms and the different families of angiosperms are indicated.

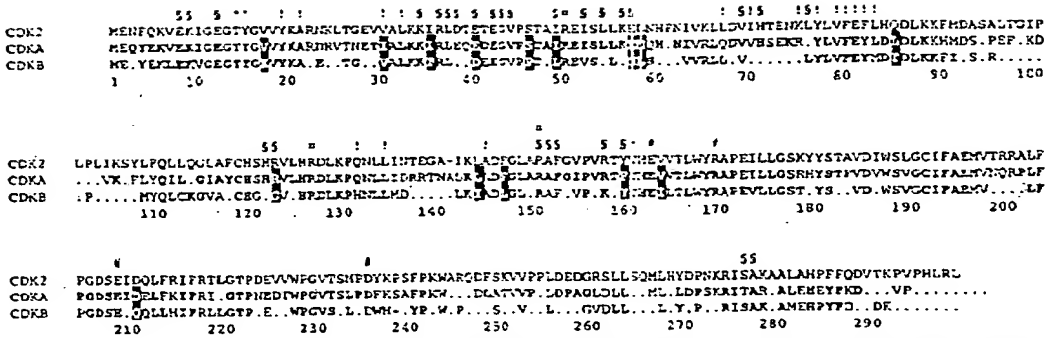

Figure 5. Comparison of the sequence and functional characteristics of human CDK2 with the consensus sequence of plant CDKA and CDKB kinases. Annotations above the CDK2 sequence refer to functional data concerning the CDK2 human protein: *, residues that are subject to regulatory phosphorylation; ■, basic residues putatively involved in stabilizing the phosphorylated T-loop (Thr-160) by ionic interactions (Russo *et al.*, 1996b); #, residues involved in interacting with the conserved SPXK sequence motif of the protein substrate (where S is the phosphoacceptor) (Russo *et al.*, 1996b); §, residues involved in binding cyclin A (Ducommun *et al.*, 1991; DeBondt *et al.*, 1993; Jeffrey *et al.*, 1995); !, residues involved in binding the inhibitor $p27^{KIP1}$ (Russo *et al.*, 1996a). The consensus sequences of CDKA and CDKB were established with 31 and 7 sequences from higher plants respectively (Table 1). The residues are indicated when they are similar in at least 80% of the CDKA sequences or in all of the CDKB sequences. Residues in bold type are identical in all the sequences of a given group. The 15 conserved residues which are different between CDKA and CDKB are in reverse print. The dots represent non-conserved residues and dashes have been introduced for maximum alignment.

kinases could be associated to CycD cyclins (Sorrell *et al.*, 1999).

However, the fact that CDKA kinases can bind cyclins other than the CycD family remains an open possibility. Site-directed mutagenesis and crystallographic studies indicate that ca. 28 residues of human CDK2 are bound to cyclin residues in the CDK2-cyclin A complex (DeBondt *et al.* 1993; Jeffrey *et al.*, 1995) (Figure 5). The fact that plant CDKA kinases possess 23 of these 28 residues may indicate the formation of a CDKA-CycA complex. Fewer residues are conserved in CDKB (18, Figure 5) and in human CDK6, which bind only cyclin D (12, data not shown). However, a few conservative substitutions amongst these residues, notably in the T-loop, have already been remarked between plant CDKA and human CDK2, and may be indicative of a specific requirement for binding plant cyclins (Dudits *et al.*, 1998). Finally, the *Arabidopsis* CDKA;1 protein was shown to bind *S. pombe* $p13^{Suc1}$, human CksHs1 and *Arabidopsis* Cks1At homologues (Deveylder *et al.*, 1997).

Inhibitors of the Kip/Cip family, such as $p27^{Kip1}$, bind human CDK2 mostly in the N-terminal lobe, with 16 residues involved in the binding (Russo *et al.*, 1996a). There is roughly the same number of these residues, ca. 10-12, conserved in CDKA and CDKB kinases, and also in human CDK6 which is also able to bind $p27^{KIP1}$ (Figure 5) suggesting that the plant kinases can bind to plant homologues of the Kip/Cip family. This has recently been confirmed by the characterization of a family of *A. thaliana* CKI named ICK1, -2, -3 with an important, although limited to a short domain, consensus sequence shared with $p27^{KIP1}$ (Wang *et al.*, 1997, 1998). The ICK1 protein has been shown by *in vitro* methods to bind both Arath;CDKA;1 and Arath;CycD3;1 (Wang *et al.*, 1998), a further indication for CDKA-CycD complexes in plants.

The INK4 family of inhibitors are specific for animal CDK4 and CDK6 kinases, which have no homologues in plants. Seven residues have been reported to account for the specificity of binding of $p19^{INK4}$ to CDK6 compared to CDK2 (Brotherton *et al.*, 1998; Russo *et al.*, 1998). It is significant that both CDKA and CDKB (see below) kinases display 6 differences amongst these residues as compared with human CDK6 (data not shown). This is an indication for the absence in plants of the INK4 family of CKI.

Plant CDKB

Seven plant CDK form a class that has the particularity of including only plant sequences. They can be identified at the position of helix α1 by a PPTALRE or PPTTLRE motif, the two bound prolines constituting

a plant-specific hallmark among CDK-related kinases. As these sequences are found in 5 families of monocotyledons and dicotyledons, they quite likely represent a plant-specific, evolutionarily conserved gene class for which the name *CDKB* is proposed (Figure 2) (Burssens *et al.*, 1998; Mironov *et al.*, 1999). Plant *CDKB* are unable to complement yeast *cdc2/cdc28* mutants (Imajuku *et al.*, 1992; Fobert *et al.*, 1996). The expression pattern of *CDKB* at the transcriptional, translational and activity level is dependent on the cell cycle phase, in difference to *CDKA* (for a review, see Mironov *et al.*, 1999) (Figure 3).

Two groups of higher-plant *CDKB* are apparent from study of the tree. Three sequences from *A. thaliana* (cdc2bAt), *M. sativa* (cdc2MsD) and *A. majus* (cdc2cAm) cluster in one branch and are characterized by a PPTALRE motif. Three other sequences from *M. sativa* (cdc2MsF), *A majus* (cdc2dAm) and *Mesembryanthemum crystallinum* (cdc2Mc) cluster in another branch and display a PPTTLRE motif. The protein sequences in the two groups display conservative differences in two regions: the α1-L5-β4 region and the L8-α3 region. The fact that both groups contain sequences from three different species and that in two genera, *Antirrhinum* and *Medicago*, *CDKB* are found in both groups may be a strong indication that the *CDKB* class encompasses two evolutionarily conserved and thus functionally different groups of genes. In accordance with the guidelines of the Commission of Plant Gene Names (Price *et al.*, 1996) and with the previously established plant cyclin nomenclature (Renaudin *et al.*, 1996), we propose to identify these as *CDKB1* and *CDKB2*, corresponding to the PPTALRE and to PPTTLRE motifs respectively, in dicotyledons. The genes from these two groups differ slightly in the timing of expression during the cell cycle: *CDKB1* transcripts accumulate during S, G_2 and M phases, whereas *CDKB2* expression is specific to G_2 and M phases (Fobert *et al.*, 1996; Segers *et al.*, 1996; Magyar *et al.*, 1997; Umeda *et al.*, 1999). The amount of CDKB protein follows the pattern of transcription and the maximum kinase activity is obtained during M phase.

The single CDKB sequence from rice, a monocotyledon, has a PPTALRE motif but appears more related to CDKB2 than to CDKB1. A unique algal CDK sequence from *Dunoliella tertiolecta*, although distant from higher plants, appears to be also related to the CDKB group and displays a unique PSTTLRE motif. The final characterization of the organization of the CDKB class in plants and the precise consensus sequences of CDKB1 and CDKB2 awaits the identification of numerous additional sequences, notably from monocotyledons and from lower plants.

The primary structure of plant CDKB kinases displays a number of conserved elements compared with the kinases from the PSTAIRE class (Figure 5). The ATP-binding site, the residues involved in regulatory phosphorylation and the definition of the consensus phosphoacceptor site are remarkably identical between the two groups of kinases. However, a number of conserved structural changes are indicative of the functional specificity of plant CDKB. There are as many as 16 changes, instead of 3 in CDKA, between CDKB and human CDK2, amongst the 38 conserved residues exposed to solvent in free human CDK2 (data not shown). Plant CDKB are characterized by 15 conserved differences as compared with plant CDKA, which are indicated in reverse print in Figure 5. A semi-conservative change was notably found in subdomain VII at the highly conserved F^{146} residue of human CDK2, which is invariant in nearly all eukaryotic serine-threonine and tyrosine protein kinases and which is replaced by a leucine residue in CDKB. The position of this residue at the boundary between the catalytic cleft and the T-loop is indicative of its role in kinase function and regulation. No data are available about the cyclin partners of CDKB, although it may be supposed that CDKB proteins bind plant cyclins expressed at the same timepoint (Figure 3; see also Figure 1 in Mironov *et al.*, 1999). CDKB kinases have only 18 over the 28 residues of human CDK2 involved in cyclin A binding, which may be an indication of preferential binding to plant cyclins B (CycB). The CDKB kinases display the same characteristics as CDKA with respect to the sites involved in binding to CKI, as deduced from animals (see above). The *Arabidopsis* CDKB;1 protein is able to bind Cks1At and its human homologue Cks1Hs, although weakly (Mironov *et al.*, 1999).

Plant CDKC

A small group of four plant CDK from pea, alfalfa and *Arabidopsis* was characterized by the presence of the PITAIRE motif, also present in the human CDK-related CHED kinase (Lapidot-Lifson *et al.*, 1992) and in the *Caenorhabditis elegans* CDK9-related kinase (Figure 2). The human CDK9 kinase, which has a PITALRE motif (Defalco and Giordano, 1998), also belongs to this class (data not shown). It is proposed that plant PITAIRE *CDK* genes be named *CDKC*

in accordance with the representatives of this family in alfalfa (cdc2MsC) and *Arabidopsis* (cdc2cAt and cdc2dAt, both identified as expressed sequence tags; Burssens *et al.*, 1998). The expression profile of the representative members of alfalfa *CDKC* was constitutive in a synchronized cell suspension (Magyar *et al.*, 1997) (Figure 3). Mironov *et al.* (1999, pp. 510–511) argued against the involvement of *CDKC* in cell cycle control since no *in situ* hybridization signal for an *Arabidopsis* member of the *CDKC* family could be obtained in actively dividing cells. The function of these kinases will be understood when their enzymatic activity can be assayed during the cell cycle and when their cyclin regulator and their substrates are known. It may be a functional indication that the human CDK9 kinase is involved in the control of transcription (Defalco and Giordano, 1998).

Plant CDKD

Two plant sequences, *A. thaliana* CAK2 and rice R2, fall into one group which also includes the Crk1/Mop1 sequence from *S. pombe* and the CDK7 sequences from *Xenopus laevis*, *Homo sapiens*, and *Dictyostelium discoidum*. The CDK7 kinases are considered to be bifunctional proteins involved in phosphorylation-dependent activation of other CDK during the cell cycle (i.e. CAK function) and in phosphorylation-dependent regulation of the activity of RNA polymerase II (Harper and Elledge, 1998). The functionality of rice *CDKD;1* was demonstrated by complementing a CAK-deficient mutant of budding yeast with the rice cDNA (Yamaguchi *et al.*, 1998). Furthermore, it was shown that rice CDKD;1 protein was able to phosphorylate not only the rice CDKA;1, but also the human CDK2 and the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II from *Arabidopsis* like other CDK7 kinases (Serizawa *et al.*, 1995). It is proposed that the *CDK7*-related plant genes be named *CDKD*. These kinases have a conserved N(I/F)TALRE motif closely related to the equivalent motif of CDK7 kinases, with a conserved asparagine residue. It should be emphasized that plants appear to possess a second kinase, which displays the function of CAK in addition to CDK7-related kinases. This kinase is named CAK1 in *A. thaliana* (Umeda *et al.*, 1998). In *S. cerevisiae*, an unusual kinase was identified as CAK which had no CTD-kinase activity, although its amino acid sequence was distinct from that of *Arabidopsis* CAK1 (Espinoza *et al.*, 1996; Thuret *et al.*, 1996; Kaldis *et al.*, 1999).

In partially synchronized suspension cells (Sauter, 1997) and during adventitious root growth (Lorbiecke and Sauter, 1999), a preferential expression of the rice *CDKD;1* gene was recorded in G_1 and S phases. At the cellular level, *CDKD;1* was shown to be uniformly associated with the dividing region of the rice root apex (Umeda *et al.*, 1999), but a basal level of expression was detected in the differentiated zone of the internode, i.e. in cells at the G_0 state (Sauter, 1997). These preliminary results indicate that *CDKD* mRNA may be slightly more abundant in G_1 and S phases, suggesting their involvement in cell-cycle regulation.

Plant CDKE

A unique plant sequence, the alfalfa cdc2MsE, appears unrelated to any other plant sequence as it harbours a SPTAIRE motif. The most similar although distantly related protein, with the sequence SMSACRE in α1 helix, is human CDK8 which is involved in the regulation of RNA polymerase II in association with cyclin C (Tassan *et al.*, 1995; Rickert *et al.*, 1996) (Figure 2). We propose naming plant *CDK* genes still to be isolated and related to the alfalfa cdc2MsE, *CDKE*. The involvement of *CDKE* genes in the plant cell cycle has yet to be proved as it displays at the mRNA level a weak constitutive signal during a synchronized cell cycle (Magyar *et al.*, 1997).

Conclusion

The high level of conservation of molecular mechanisms in the eukaryotic cell cycle is exemplified by the CDKA class of plant CDK-related kinases, which display a large number of structural and functional properties very similar to those of their human and yeast counterparts. However, the specificity of the plant cell cycle may be appreciated by the fact that plant CDKA kinases form complexes with plant homologues of D-type cyclins. In animals, binding to cyclin D is a property of CDK4 and CDK6 kinases, which are absent from the plant kingdom. Hence, common cell cycle tools may be used in different organisms and in various combinations to achieve cell cycle regulations. However in plants, these mechanisms have still to be described precisely at the molecular level.

The peculiarities of the plant cell cycle with respect to other eukaryotes may also account for the occurrence of a plant-specific class of CDK, CDKB, encompassing two different gene families: CDKB1

and CDKB2. Conversely, classes found in other metazoans, such as CDK4 and CDK6 or CDK5, are absent in plants. This and the above data justify plant-specific cell cycle studies, although the basic conservation of cell cycle mechanisms still allows the deduction of plant CDK functions from animal and yeast counterparts.

The nomenclature proposed in this review seeks to replace a number of current nomenclatures (Mironov *et al.*, 1999), of which the most prevalent is the independent naming of CDK in each species. The advantages of this system have been detailed previously for plant cyclins (Renaudin *et al.*, 1996). Much remains to be done at the biochemical level to identify the proper combinations of plant CDK and cyclin, their regulation by CKI and their substrates. It is expected that establishing a common nomenclature for cyclins and CDK will provide a valuable tool and allow progress in our understanding of plant cell cycle controls.

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